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(54) Villin gene promoter sequence and its use in vectors, transformed mammalian cell lines, transgenic animals, and cell lines derived from the animals.

(57) An isolated DNA sequence encoding a promoter of the human villin gene is provided. The DNA sequence can be operatively linked to a nucleotide sequence of a gene, such as an oncogene, and incorporated in cloning and expression vectors. The vectors are useful in the production of transgenic, non-human mammals as models of colorectal cancer.

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Technical Fi ld

This invention relates to a gene promoter sequence for r gulating gen expression in mammalian cells. This invention also relates to v ctors containing the promoter sequenc , cells transformed with the vectors, transgenic animals based on the vectors, and cell lines derived from cells in the animals.

Background Art

The ability to introduce genes into the germ line of mammals is of great interest in biology. The propensity of mammalian cells to take up exogenously added DNA and to express genes included in the DNA has been known for many years. The results of gene manipulation are inherited by the offspring of these animals. All cells of these offspring inherit the introduced gene as part of their genetic make-up. Such animals are said to be transgenic.

Transgenic mammals have provided a means for studying gene regulation during embryogenesis and in differentiation, for studying the action of oncogenes, and for studying the intricate interaction of cells in the immune system. The whole animal is the ultimate assay system for manipulated genes, which direct complex biological processes.

Transgenic animals can provide a general assay for functionally dissecting DNA sequences responsible for tissue specific or developmental regulation of a variety of genes. In addition, transgenic animals provide useful vehicles for expressing recombinant proteins and for generating precise animal models of human genetic disorders.

By means of recombinant techniques, it is possible to make constructs containing oncogenes. Many oncogene constructs have been introduced into transgenic mice and have been found to elicit tumorigenic responses. For example, mice that carry the SV40 enhancer and region coding for the large T-antigen reproducibly develop tumors of the choroid plexus, which are derived from the cells lining the ventricles of the brain. Palmiter et al., Nature **316**:457-60 (1985). In similar experiments, the *c-myc* oncogene was fused to the LTR of mouse mammary tumor virus. In one transgenic line, females characteristically developed mammary carcinomas during the second or third pregnancy. Stewart et al., Cell **38**:627-37 (1984). In other experiments, the *c-myc* oncogene was driven by immunoglobulin enhancers and gave rise to malignant lymphoid tumors in transgenic mice. Adams et al., Nature **318**:533-8 (1985). When an oncogenic human *ras* gene was fused to an elastase promoter/enhancer construct, transgenic mice were born with pancreatic neoplasms that appeared to be due to transformation of all of the differentiating pancreatic cells. Palmiter et al., Ann. Rev. Genet. **20**:465-99 (1986).

For a general discussion of gene cloning and expression in animals and animal cells, see Old and Primrose, "Principles of Gene Manipulation," Blackwell Scientific Publications, London (1989), page 255 *et seq.*

Transgenic lines, which have a predisposition to specific tumors, are of great value in the investigation of the events leading to malignant transformation. It is well known that the efficacy of an anti-cancer treatment is dependent on identification of the tumor cells that are the primary cause of the disease. This is especially true in the detection of proliferation of tumor cells of the gastrointestinal tract or of renal origin. Indeed, cancers of the digestive tract are widespread, and there is a critical need for effective treatment for these diseases. The discovery of the effective treatments can be expedited by providing an animal model that will lead to carcinogenesis, which will enable the study of the efficacy, safety, and mode of action of anti-tumoral pharmaceutical agents.

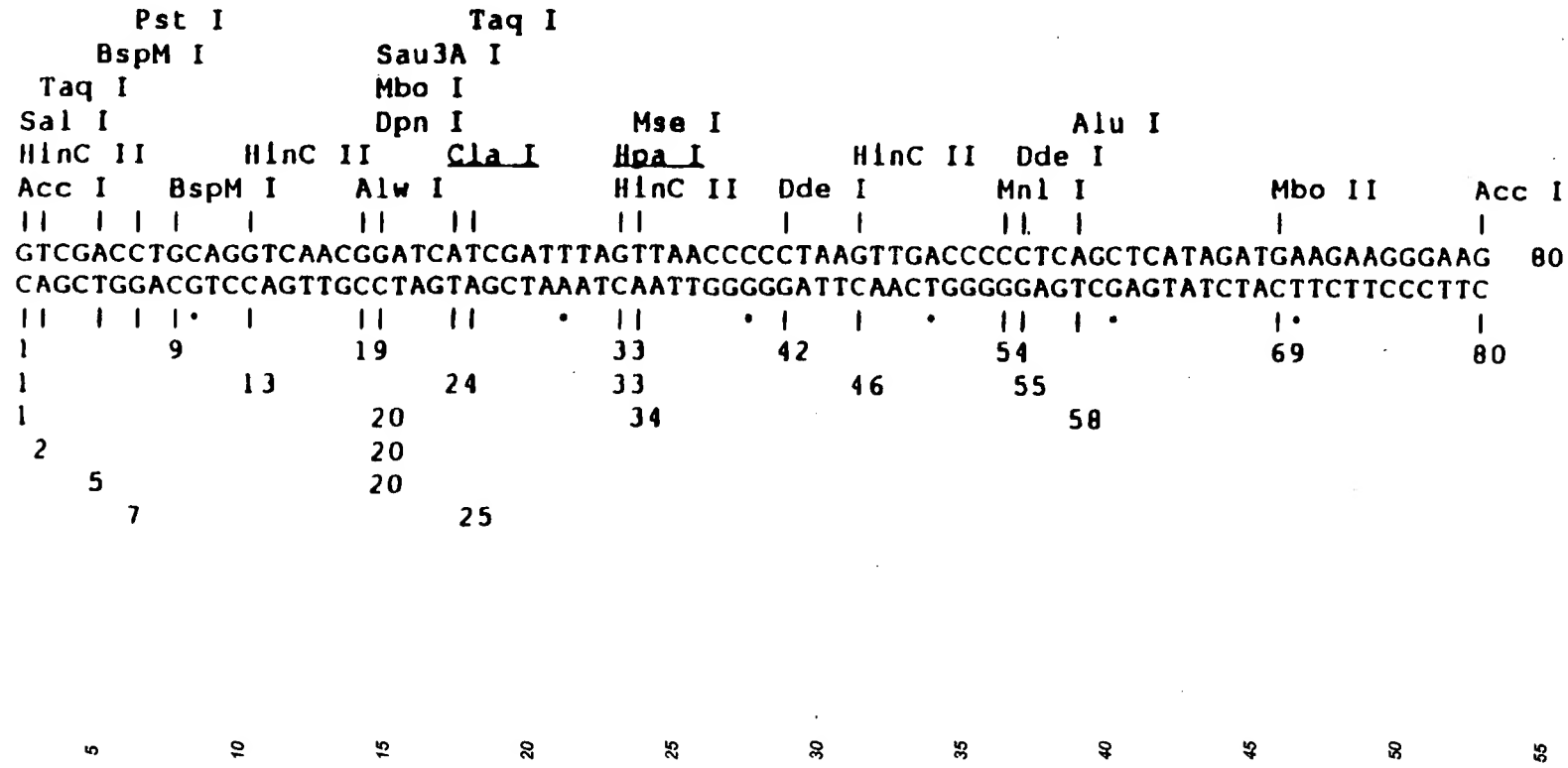
In summary, there exists a need in the art for reagents and methods for providing transgenic animal models of human tumor growth and proliferation, including transgenic animal models of human tumors of the digestive tract and renal system.

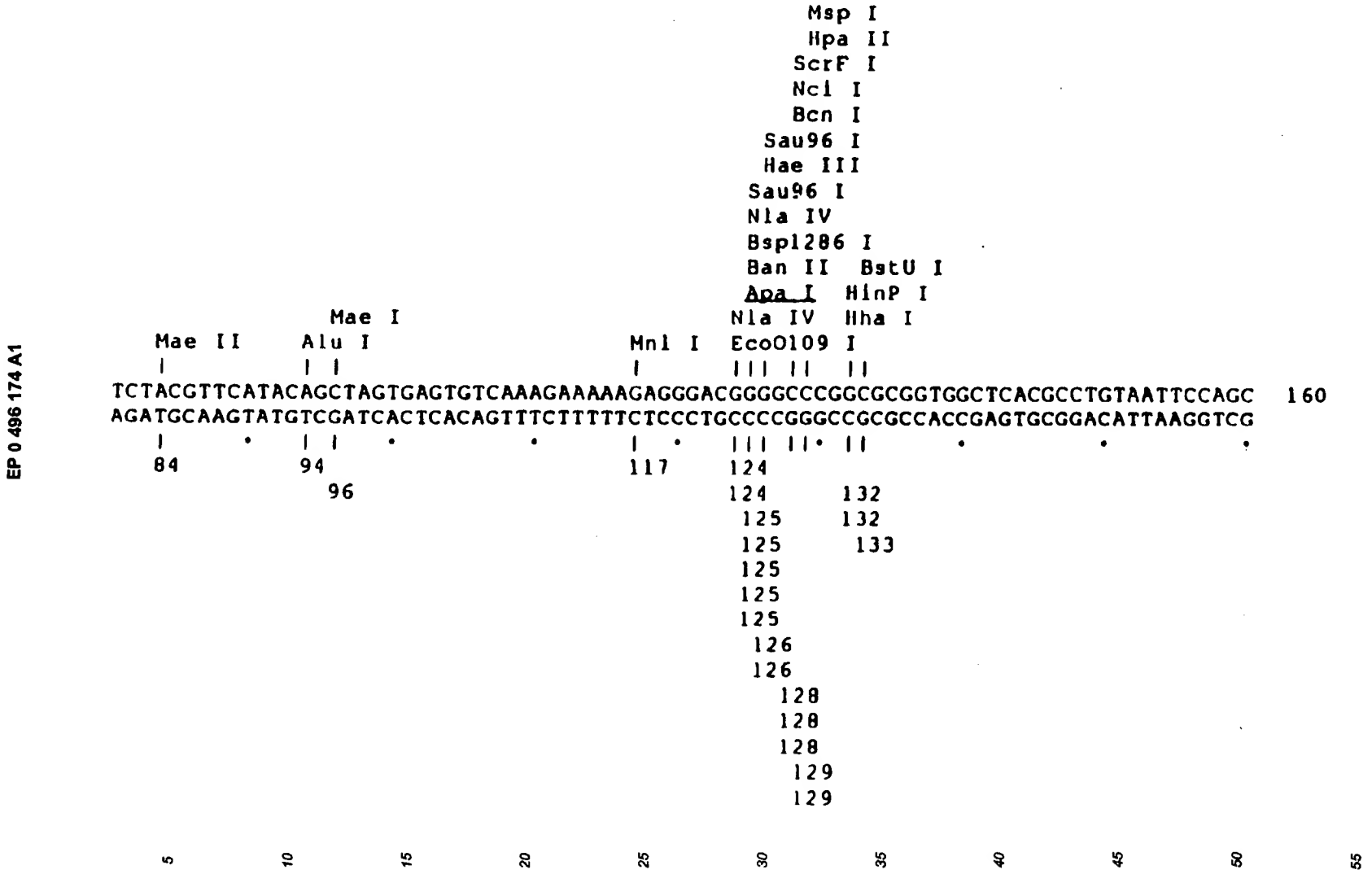
Disclosure of the Invention

Accordingly, this invention aids in fulfilling these needs in the art. Specifically, this invention provides an isolated DNA sequence encoding or corresponding to a promoter of the human villin gene. The nucleotide sequence consists essentially of :

PRV.STRIDER -> Restriction Map

DNA sequence 1991 b.p. GTCGACCTGCAG ... CTGCAGGTCGAC linear

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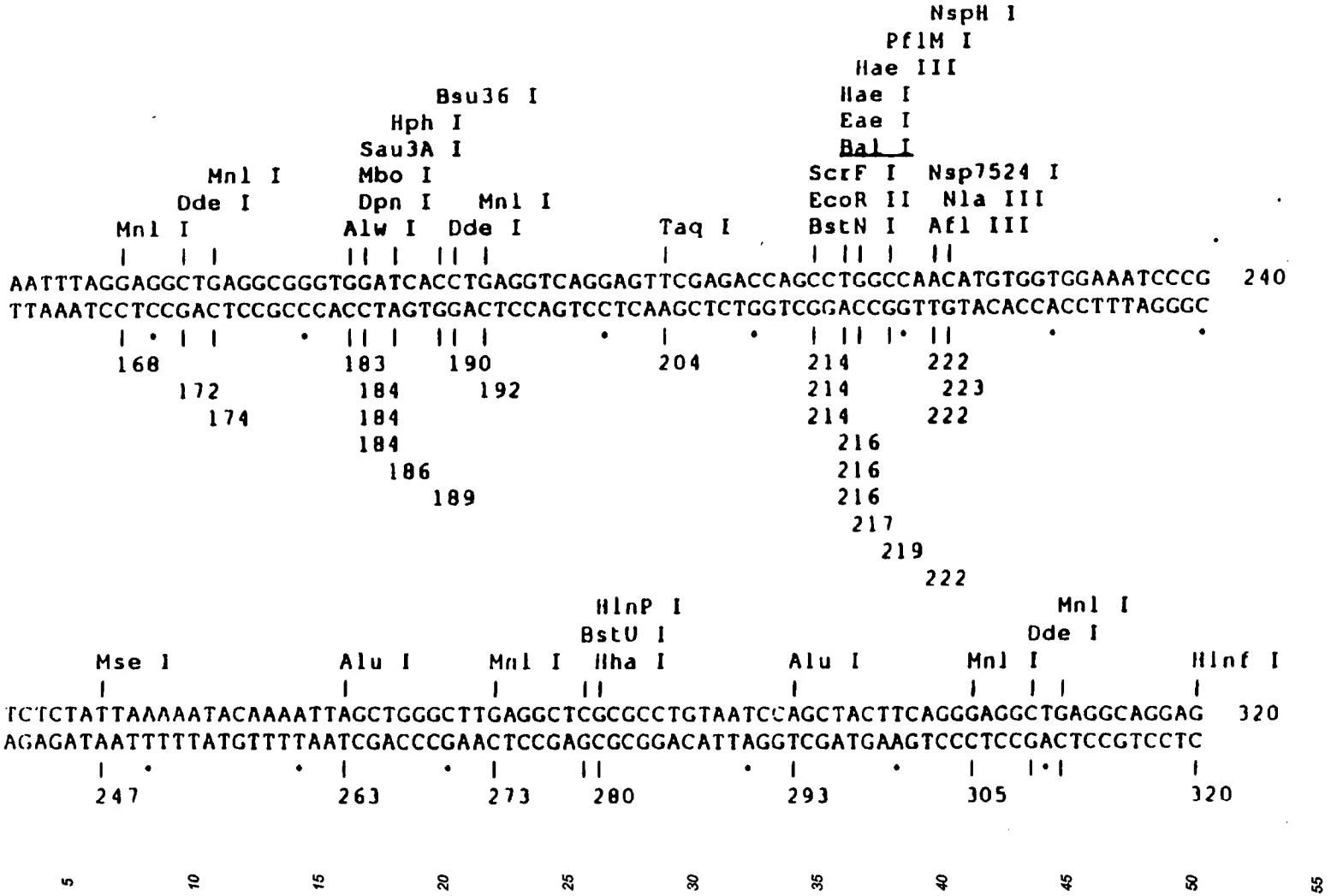
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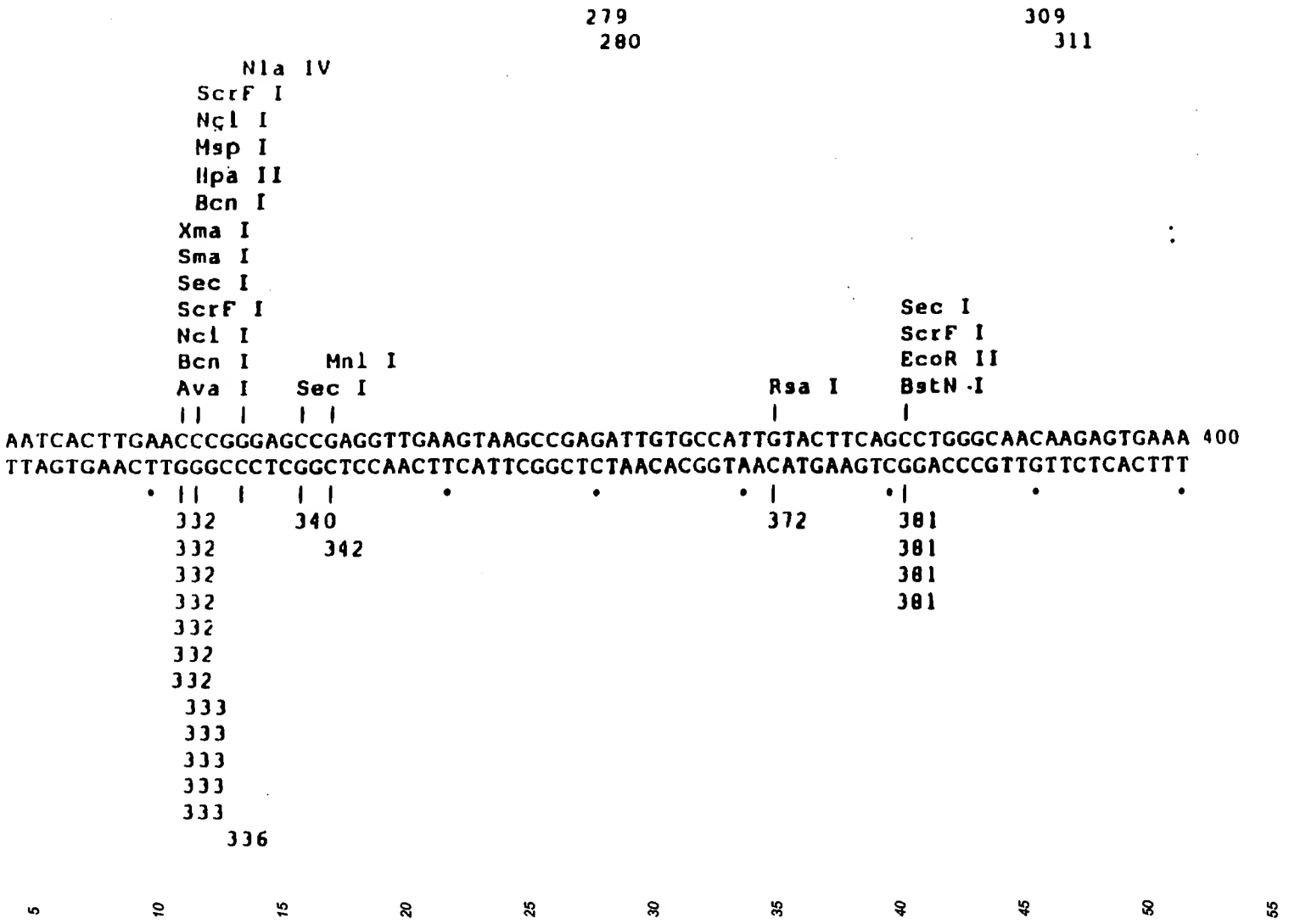
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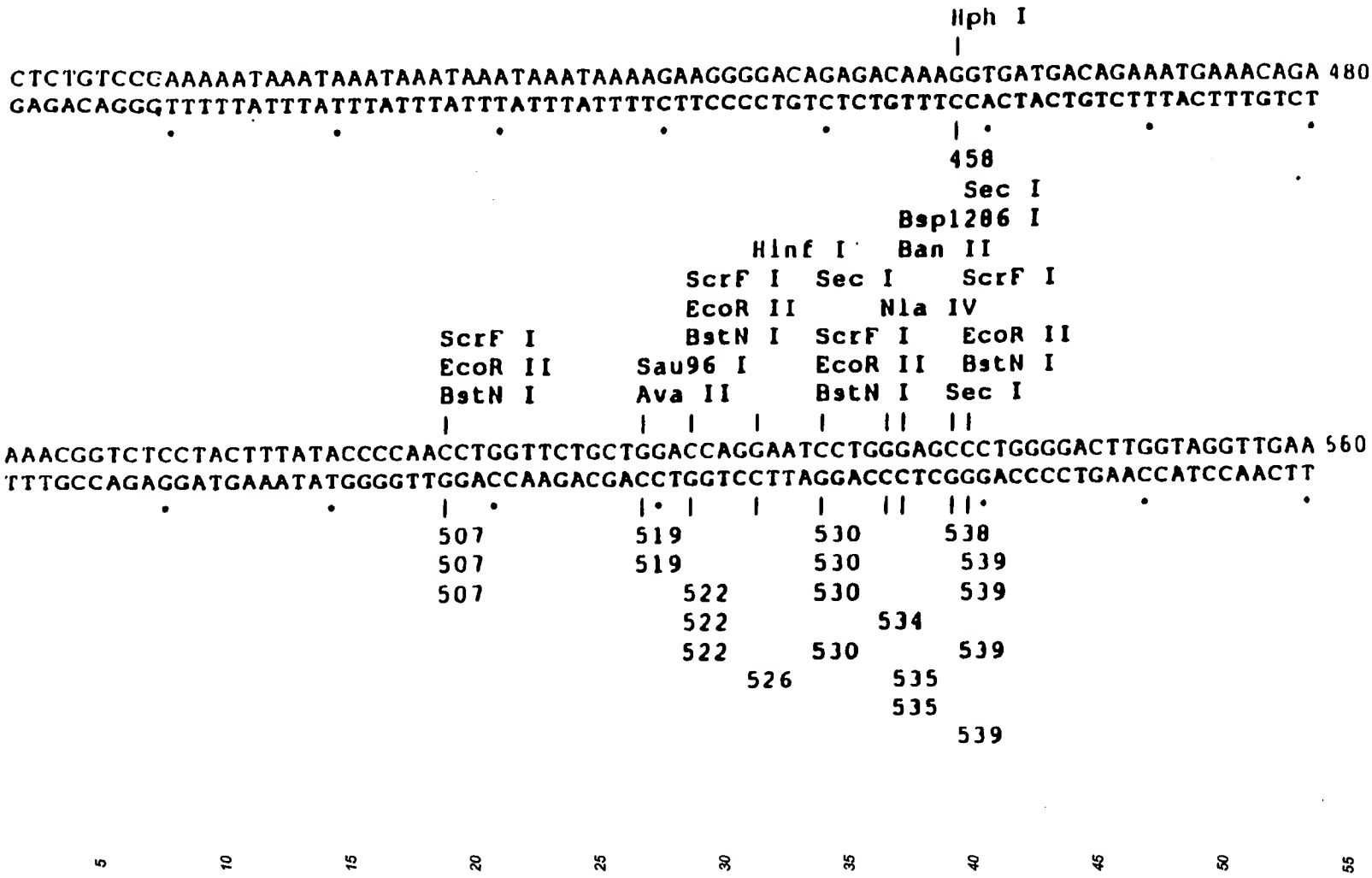
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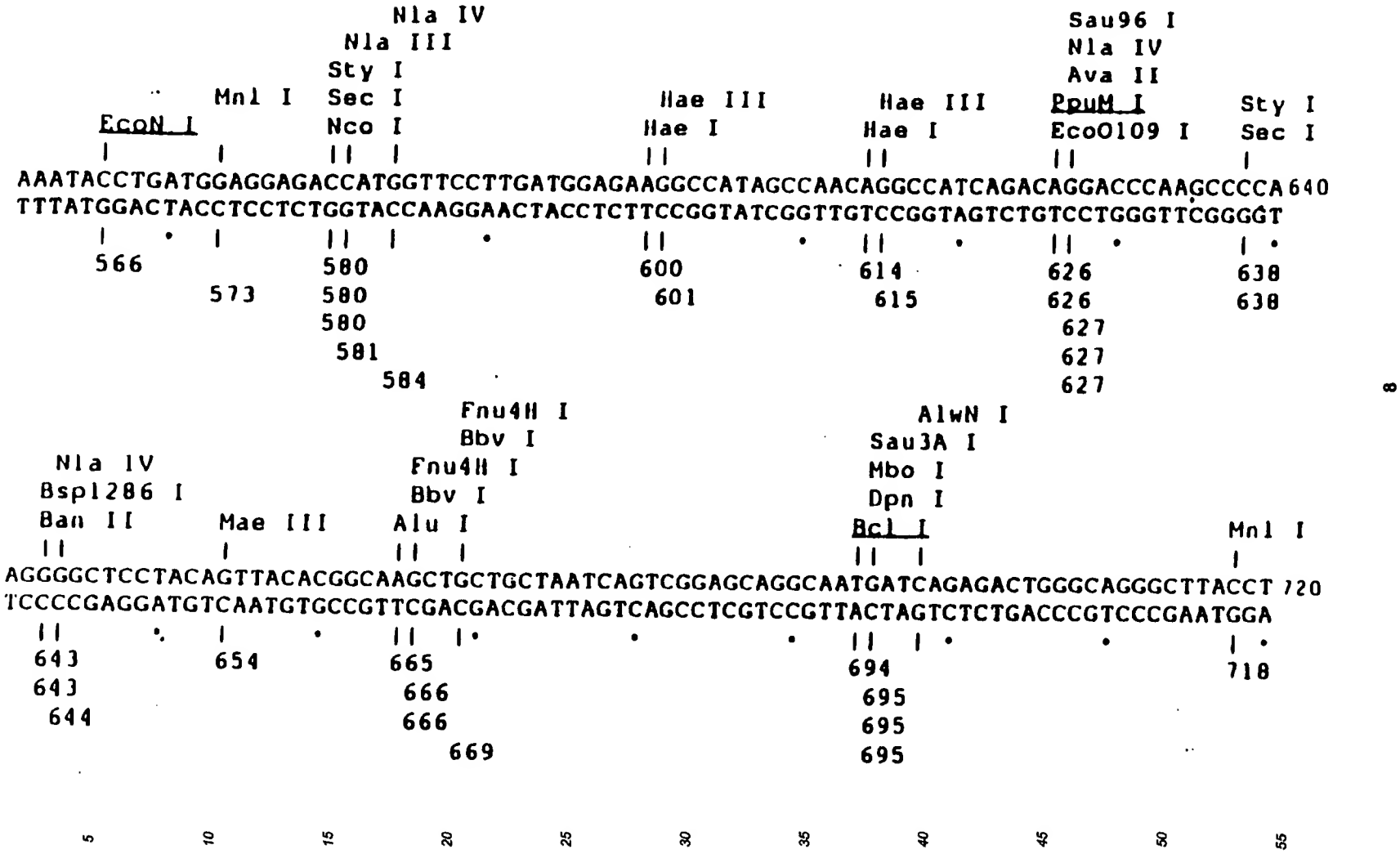
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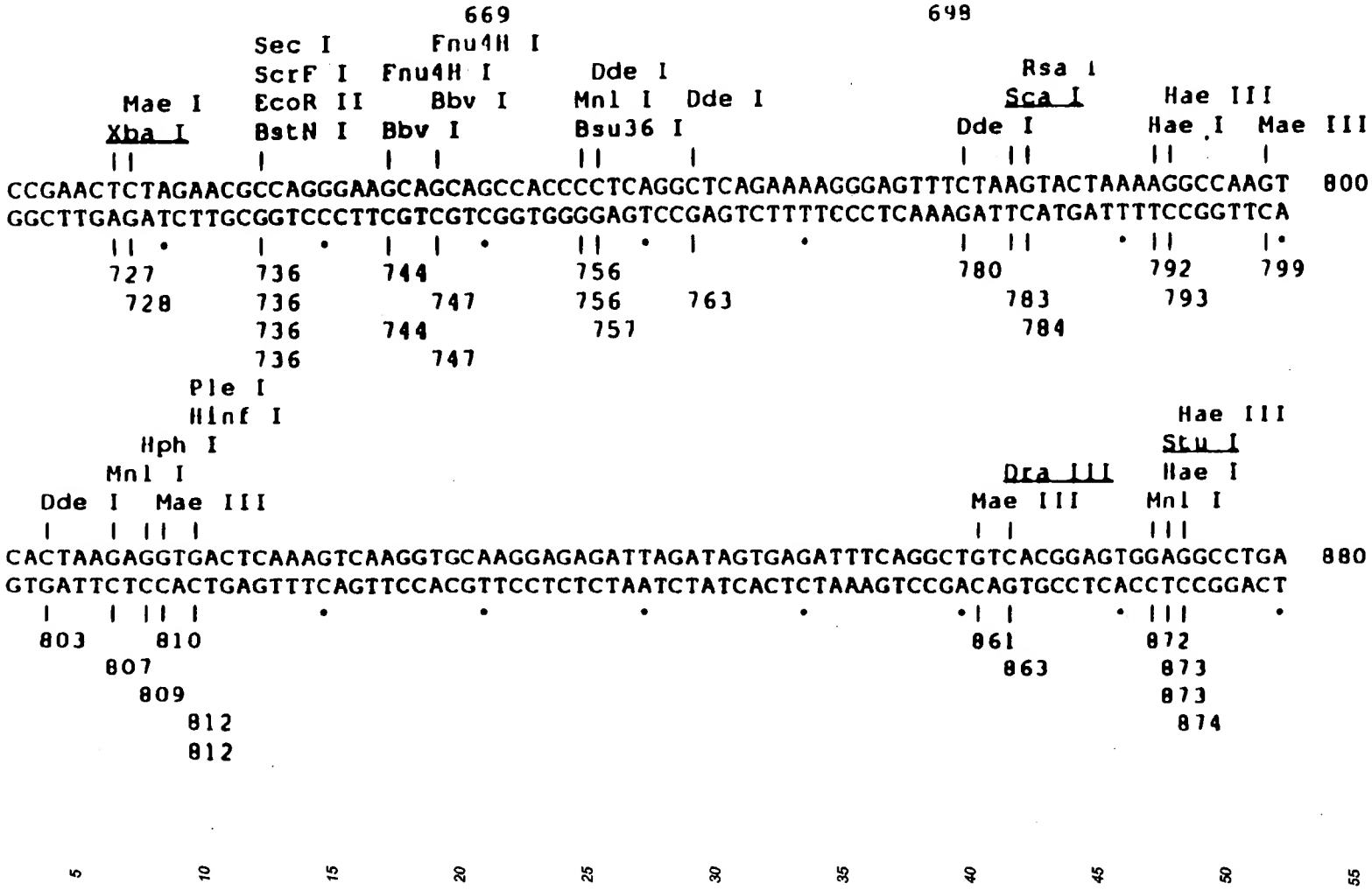
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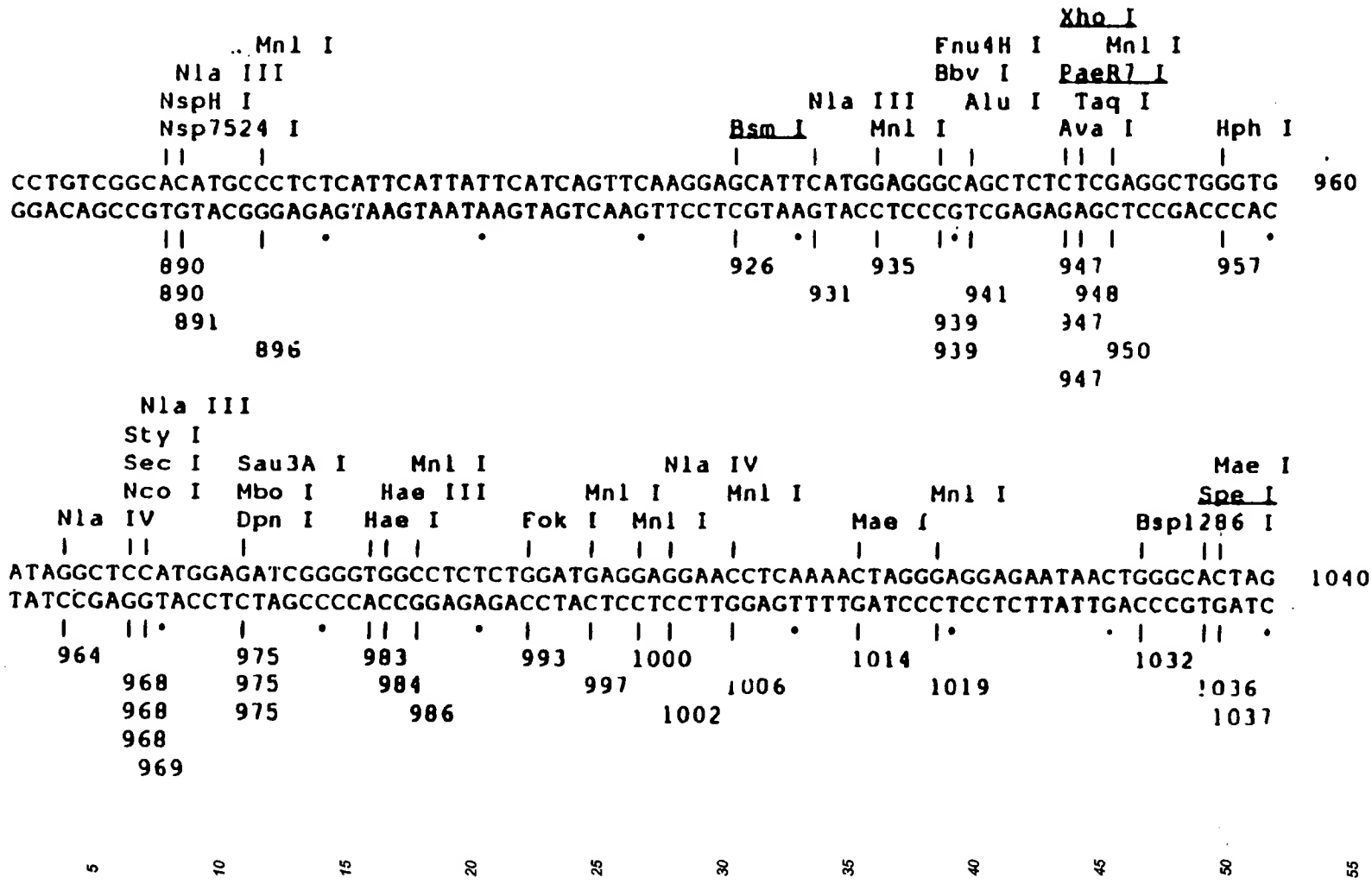
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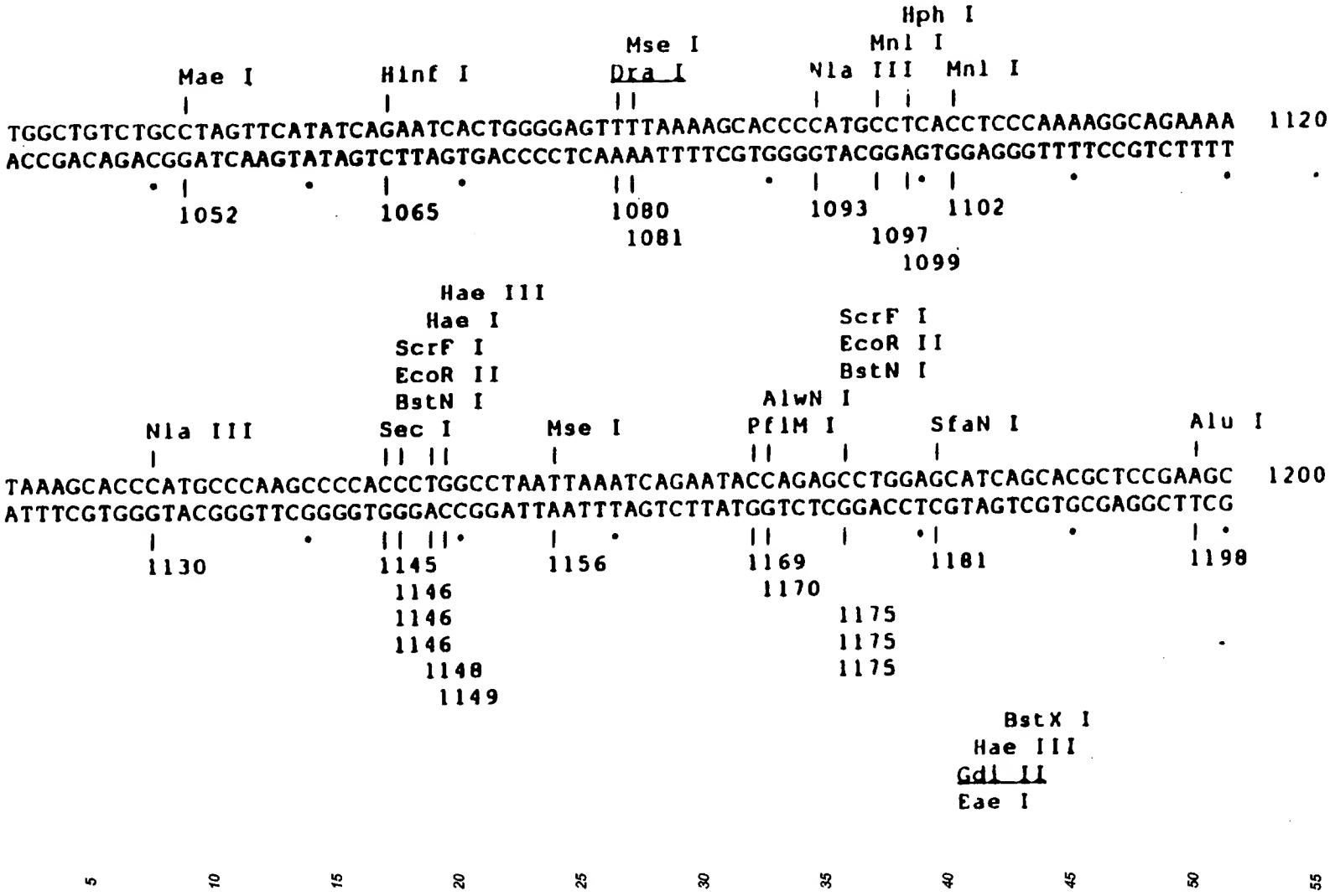
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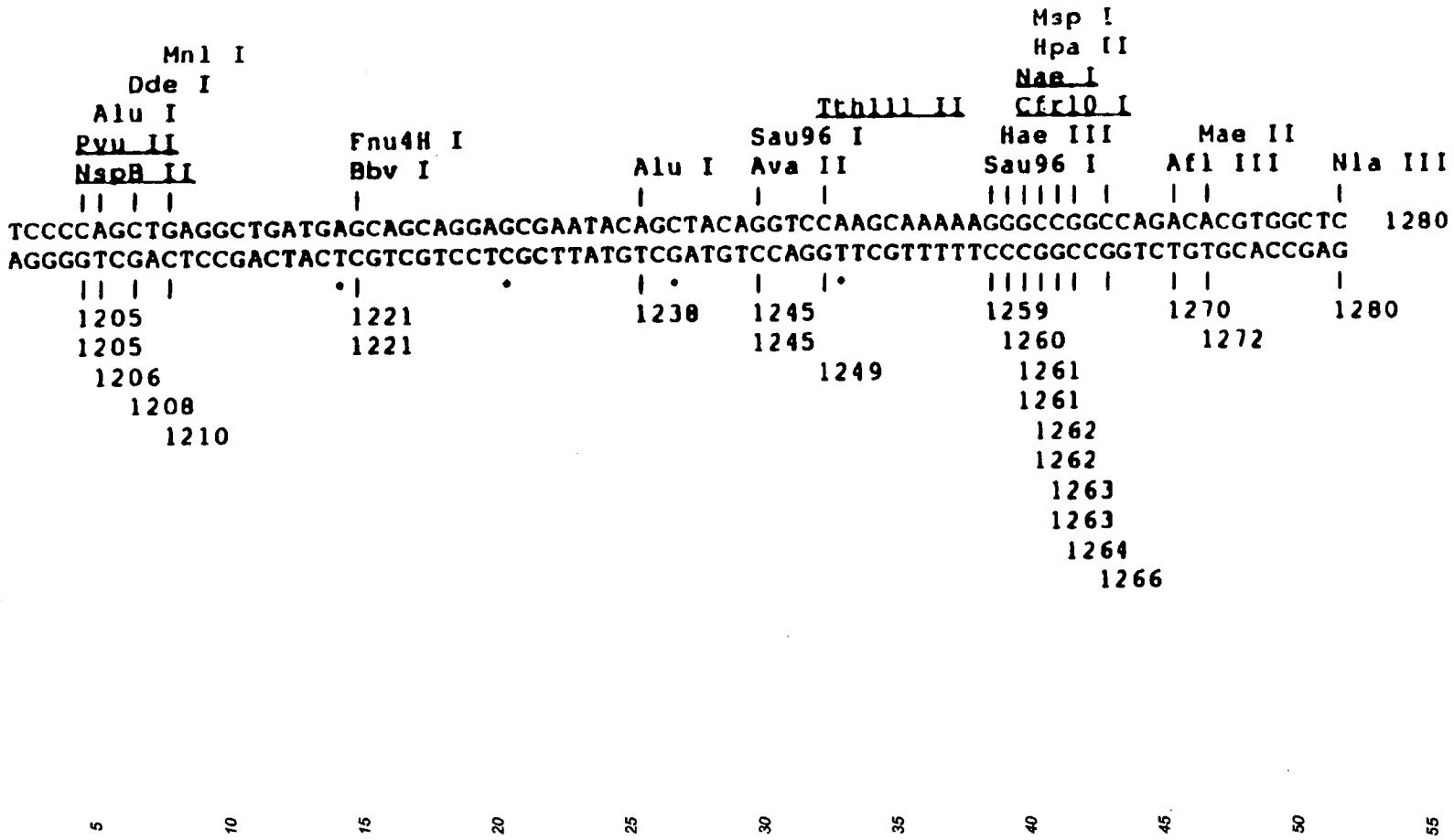
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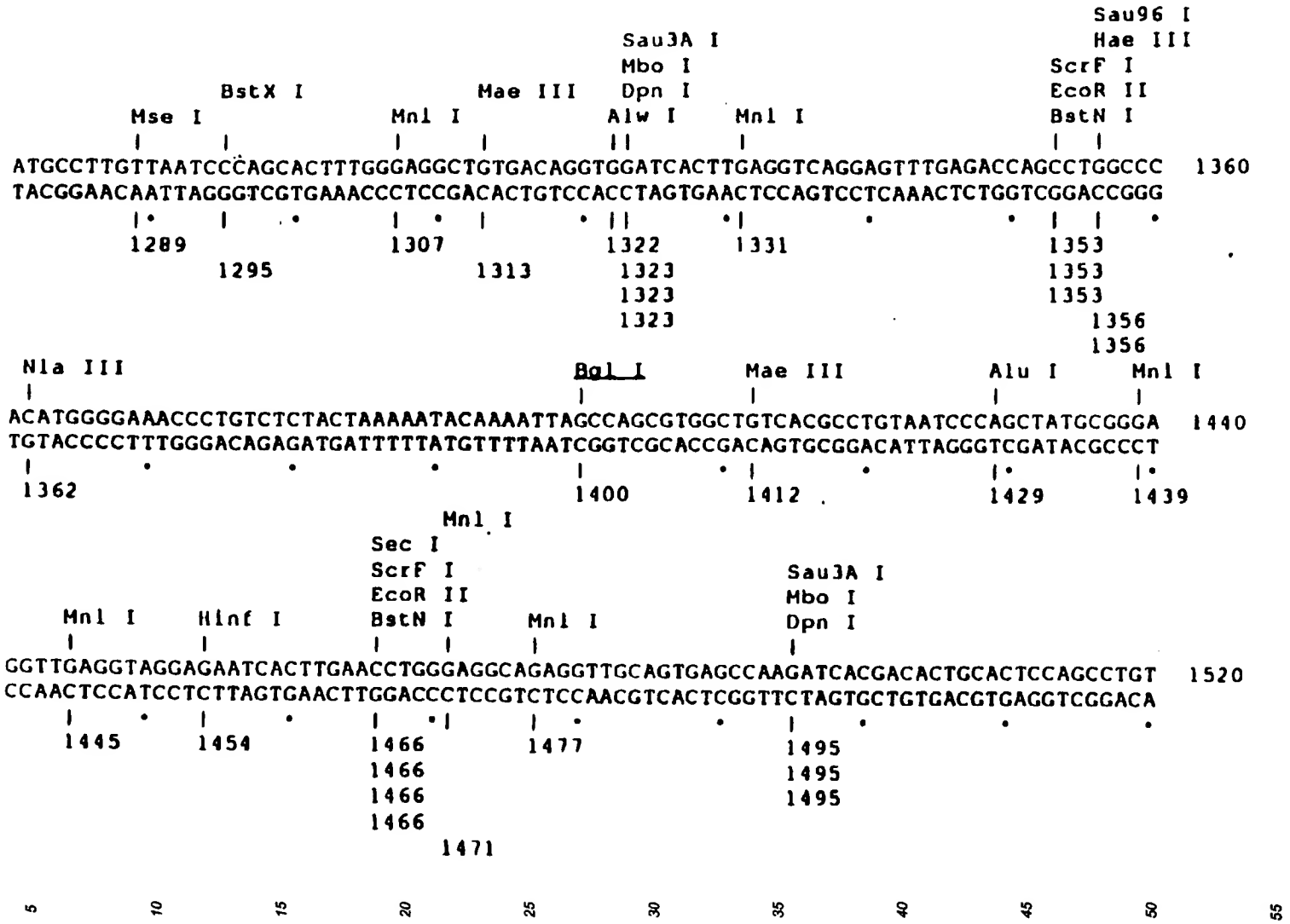
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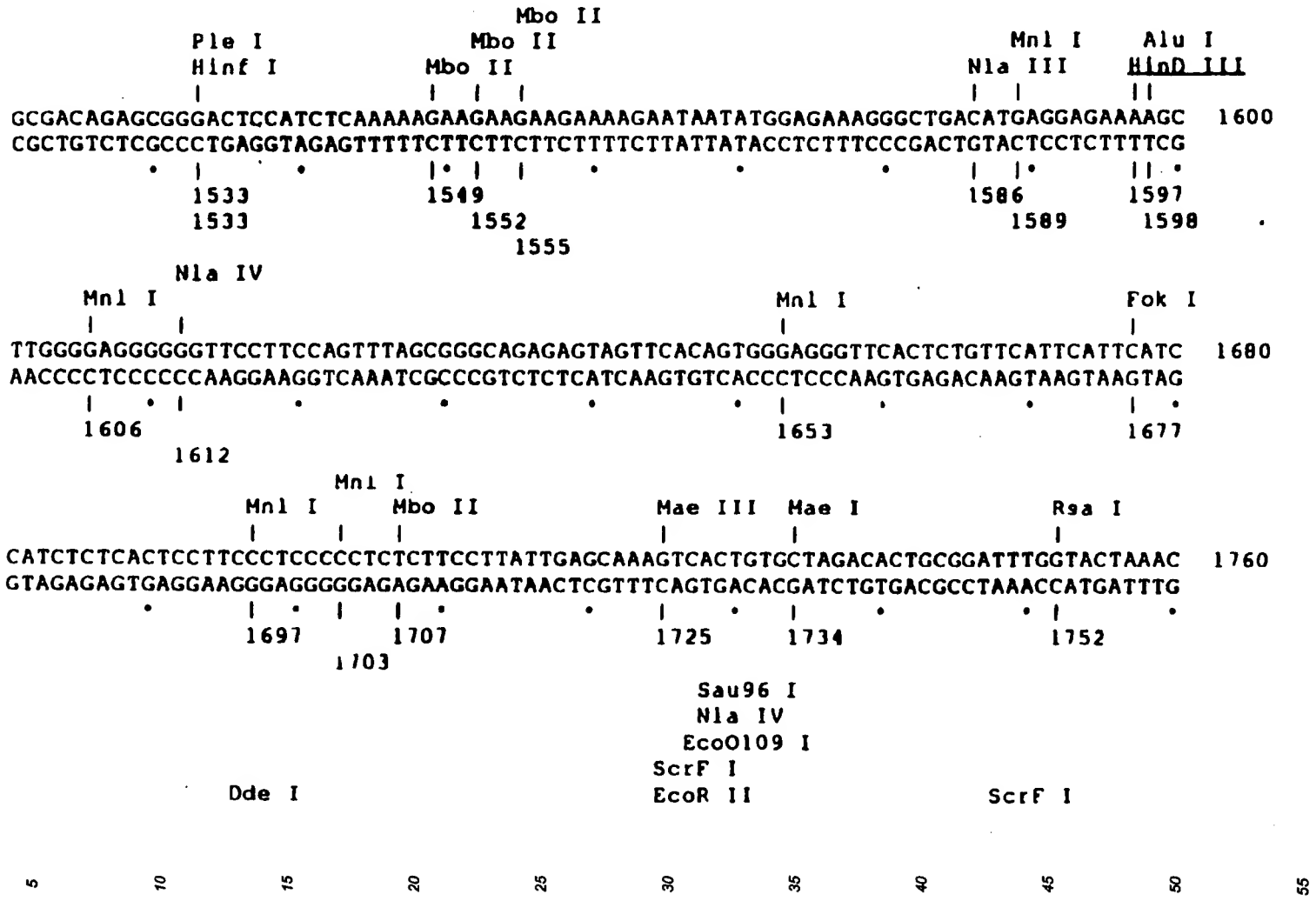
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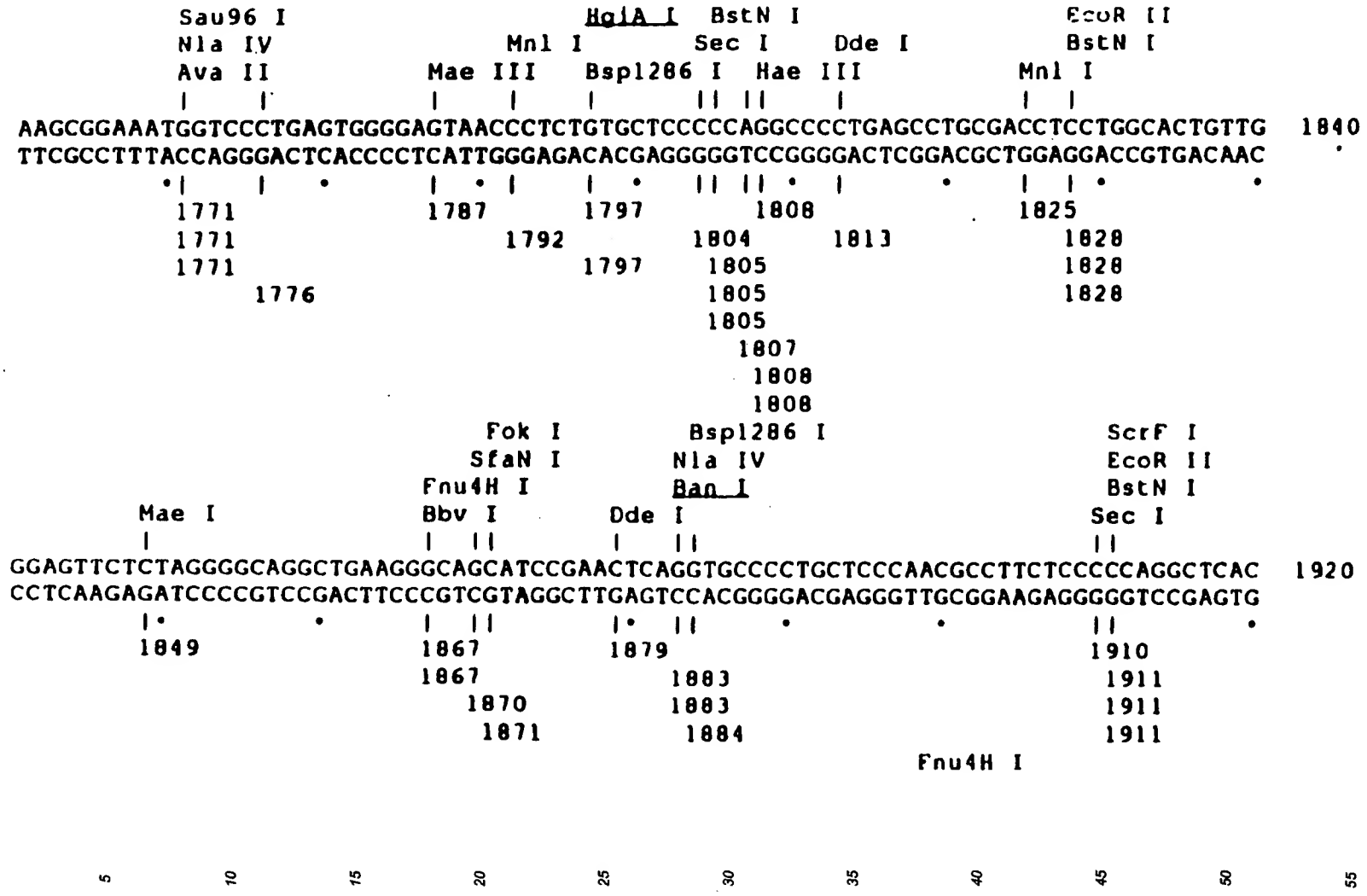
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Fok I
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Fnu4H I
Bsp1286 I
Nla IV
Ban I
ScrF I
EcoR II
BstN I
Mae I
Bbv I
Dde I
Sec I
GGAGTTCTCTAGGGGCAGGCTGAAGGGCAGCATCCGAACTCAGGTGCCCCTGCTCCCAACGCCTTCTCCCCCAGGCTCAC 1920
CCTCAAGAGATCCCCGTCCGACTTCCCGTCGTAGGCTTGAGTCCACGGGGACGAGGGTTGCGGAAGAGGGGGTCCGAGTG
1849
1867
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1870
1871
1879
1883
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1884
1910
1911
1911
1911
Fnu4H I
5
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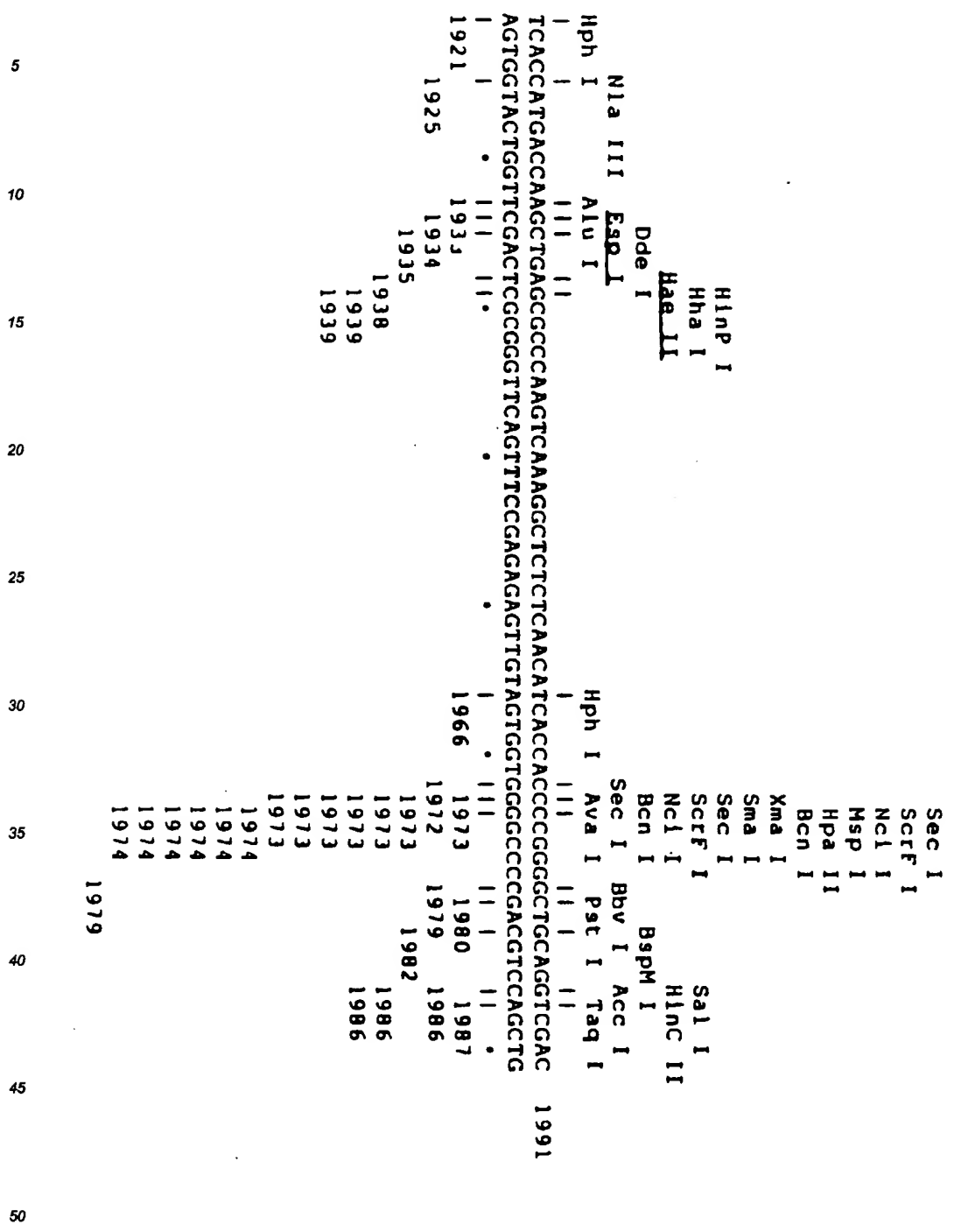

This invention also provides a DNA sequ nce comprising the promoter of th human villin gene perativ ly linked to a nucleotide sequence of a structural gene or an oncog ne. In specific embodiments of th invention, th structural gene encodes villin or a reporter gene, such as the gene coding for luciferase.

This invention further provides isolated RNA compl mentary to th promot r s quence of th human villin gene and to the other DNA sequences describ d her in.

In another embodiment of th invention, a eukaryotic v ctor is provided. Th vector comprises a plasmid

or viral vector containing the promot r s quence of the human villin g n .

In addition, this inv ntion provides mammalian c lls transformed with the vectors of th invention.

Also, this invention provides transgenic animals containing the promoter of the human villin gen and cell lines cultur d from cells of the transgenic animals.

Brief Description of the Drawings

This invention will be more fully described with reference to the drawings in which:

Fig. 1′ is a representation of the structure of the isolated 5′-flanking region of the villin gene;

Fig. 2 depicts a partial structure of the human villin gene showing the approximate location of introns and exons;

Fig. 3 depicts the nucleotide sequence of a portion of human villin cDNA, including the location of the intervening sequences (IVS) in the 5′-first 900 base pairs (bp);

Fig. 4′A-B depicts the 5′-region of the nucleotide sequence of DNA encoding a promoter of the human villin gene;

Fig. 4″A-N depicts the nucleotide sequence of the villin gene promoter, including the sequence of complementary strands and restriction endonuclease sites;

Fig. 5 depicts plasmid vector pSVOA and the luciferase gene employed in one embodiment of the invention;

Fig. 6 is a diagrammatic representation of the procedure used to construct plasmid pPrVL of the invention; and

Fig. 7 is a diagrammatic representation of the preparation of an animal model for colorectal cancers and cell cultures derived from animal tumors.

Best Mode for Carrying Out the Invention

1. Gene Promoter Sequence

This invention provides an isolated DNA sequence encoding a promoter of the human villin gene. Villin is a protein with a molecular weight of about 95,000 daltons and is normally present in the villi of the digestive mucosa, more especially in intestinal villi. Villin is able to bind to actin in the presence of calcium ions.

Villin is usually found in brush borders, which are observed at the apex of enterocytes, particularly after they have attained their final stage of differentiation. The enterocytes line the entire intestinal lumen. Micro-villi forming the brush border are assembled at the final stage of differentiation of the enterocyte. Villin is localized in bundles of axial microfilaments of micro-villi. It contributes to the structure of their cytoskeleton. On examining frozen sections by immunofluorescence, it has been observed that villin is present at the apical pole of elongated cells forming columns at the surface of the internal wall of the intestine, and also close to cells of the proximal tubules of the kidney. In both cases, their regions are contiguous with the brush border of the cells concerned.

Villin has not been detected in other types of micro-villi of various other epithelia cells when the micro-villi were not provided with a well-organized brush border. A. Bretscher et al., Exp. Cell Res., **135**:213 (1981); H. Reggio et al., "Membranes in Growth and Development" by A. Liss, New York (1982) pp. 89-105. Furthermore, it has been observed that villin is present in enterocytes from an early stage of their development, well before the organization of the brush border.

Villin can almost always be detected *in vivo* at all stages of the development of tumor cells in the digestive region, at both an early and later stages of tumorogenesis, irrespective of the state of differentiation of the cells concerned. In particularly, villin can be detected in cancers of the colon, one of the most widespread forms of cancer. Villin can also be detected in certain types of renal cancer, but has never been detected in primary tumor cells derived from cancers localized in the tissues of other organs, such as the liver, ovaries, and lungs.

The nucleotide sequence and the amino acid sequence of the COOH-terminal portion of human villin are given below:

```
LYS TRP SER ASN THR LYS SER TYR GLU ASP LEU LYS ALA GLU
AAG TGG AGT AAC ACC AAA TCC TAT GAG GAC CTG AAG GCG GAG

SER GLY ASN SER ARG ASP TRP SER GLN ILE THR ALA GLU VAL
TCT GGC AAC TCT AGG GAC TGG AGC CAG ATC ACT GCT GAG GTC

THR SER PRO LYS VAL ASP VAL PHE ASN ALA ASN SER ASN LEU
ACA AGC CCC AAA GTG GAC GTG TTC AAT GCT AAC AGC AAC CTC

SER SER GLY PRO LEU PRO ILE PHE PRO LEU GLU GLN LEU VAL
AGT TCT GGG CCT CTG CCC ATC TTC CCC CTG GAG CAG CTA GTG

ASN LYS PRO VAL GLU GLU LEU PRO GLU GLY VAL ASP PRO SER
AAC AAG GCT GTA GAG GAG CTC CCC GAG GGT GTG GAC CCC AGC

ARG LYS GLU GLU HIS LEU SER ILE GLU ASP PHE THR GLN ALA
AGG AAG GAG GAA CAC CTG TGC ATT GAA GAT TTC ACT GAG GCC

PHE GLY MET THR PRO ALA ALA PHE SER ALA LEU PRO ARG TRP
TIT GGG ATG ACT CCA GCT GCC TTC TCT GCT CTG CCT CGA TGG

LYS GLN GLN ASN LEU LYS LYS GLU LYS GLY LEU PHE *
AAG CAA CAA AAC CTC AAG AAA GAA AAA GGA CTA ITT TGA GAAG

AGTAGCTGTCCTTGTAAAGCAGTACCCTACCCTGATTGTAGGGTCTCATTTTCTCA
CCGATATTAGTCCTACACCAATTGAAGTGAAATTTTGCAGATGTGCCTATGAGCAC
AAACTTCTGTGGCAAATGCCAGTTTTGTTTAATAAATGTACCTATTCCTTCAGAAA
GATGATACCCCAAAAAAAAAAAA
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This nucleotide sequence and amino acid sequence identify a domani, which has been termed the headpiece (HP).

The nucleotide sequence coding for the terminal portion of human villin can be isolated by means of the following procedure:

- total mRNA (messenger RNAs) is prepared according to known techniques from a cell line expressing villin;
- a cDNA (complementary DNA) bank is constructed;
- these cDNAs are inserted into a vector capable of expressing the protein coded by the insert;
- by means of the recombinant vectors obtained, a bacterial strain is transformed, then conditions permitting the expression of the desired protein in the bacteria are established; and
- the recombinant clones containing the clone specific for villin is selected by means of antibodies recognizing villin.

These techniques are described in more detail in copending U.S. application Serial No. 287,658, filed December 21, 1988. The sequence of the entire human villin protein has been elucidated by Arpin et al., J. Cell Biol., 107:1759-1766 (1988) and Pringault et al., EMBO J., 5:3119-3124 (1986).

A plasmid containing the gene encoding human villin was deposited with the Collection Nationale de Cultures de Microorganismes (C.N.C.M.) of Institut Pasteur in Paris, France on November 13, 1985, under culture collection deposit Accession No. I-497.

A portion of the operon encoding the human villin gene is diagrammatically shown in Fig. 1′. More particularly, Fig. 1′ depicts the structure of an isolated 5′-flanking region of the operon for the human villin gene. Referring to the Figure, a 2 kb region is shown proximate the 5′-end. This region contains the gene promoter of the invention. The CAP site and the ATG start codon of the gene encoding human villin are also shown in the Figure.

It has b n discover d that the operon encoding human villin contains a numb r of xons and introns. The first exon is shown in Fig. 1′ and is identified as "EXON 1". A number of other exons and introns ar depicted in Fig. 2, which is a partial structur of an isolated 5′-flanking region of th op ron ncoding human villin mRNA. Exon 1 in Fig. 1′ corresponds to the first exon comprising nucleotides 1-97 in Fig. 2. Introns ar identified by triangular regions extending toward the bottom of th Figur . It will be appar nt from Fig. 2 that the op ron ncoding the human villin gene contains a number of intervening sequ nc s.

The location of the intervening sequ nces (IVS) in a 5'-first 900 bp of human villin cDNA is shown in Fig. 3. The IVS are d picted relative to th nucleotide sequence encoding human villin. Th sequ nce shown in Fig. 3 is the coding sequence (exons) of the villin gene. Once again, the CAP site and ATG start codon are shown in the Figure.

The DNA sequence of the invention encodes a promoter of the human villin gene. The DNA sequence is shown in Figs. 4'A-B and 4"A-N. Fig. 4'A-B depicts a portion of the DNA in the 5'-region of the promoter, and includes the CAP site and the ATG codon of the villin gene. Fig. 4"A-N depicts the entire 1991 bp DNA sequence encoding the promoter of the villin gene of the invention. The locations of restriction endonuclease sites in the DNA sequence are indicated in Fig. 4"A-N.

The nucleotide sequence of the promoter of the invention was derived by dideoxynucleotide sequencing. The base sequences of the nucleotides are written in the 5' ----> 3' direction. Each of the letters shown is a conventional designation for the following nucleotides:

A Adenine
G Guanine
T Thymine
C Cytosine.

The DNA sequence encoding the promoter of the human villin gene can be isolated from its natural environment. It is preferred that the DNA sequence encoding the promoter of the human villin gene be in a purified form. For instance, the promoter can be free of human blood-derived proteins, human serum proteins, viral proteins, nucleotide sequences encoding these proteins, human tissue, human tissue components, or combinations of these substances. In addition, it is preferred that the promoter sequence is free of extraneous proteins and lipids, and adventitious microorganisms, such as bacteria and viruses. The essentially purified and isolated DNA sequence encoding the promoter is especially useful for preparing nucleotide probes and expression vectors.

The promoter of the invention can also be prepared by the formation of 3' ----> 5' phosphate linkages between nucleoside units using conventional chemical synthesis techniques. For example, the well-known phosphodiester, phosphotriester, and phosphite triester techniques, as well as known modifications of these approaches, can be employed. Deoxyribonucleotides can be prepared with automatic synthesis machines, such as those based on the phosphoramidite approach. Oligo- and polyribonucleotides can also be obtained with the aid of RNA ligase using conventional techniques.

This invention of course includes variants of the DNA sequence of the invention exhibiting substantially the same properties as the promoter of the invention. By this it is meant that DNA sequences need not be identical to the sequence disclosed herein. Variations can be attributable to single or multiple base substitutions, deletions, or insertions or local mutations involving one or more nucleotides not substantially detracting from the properties of the DNA sequence as a promoter for the human villin gene. It will also be understood that the present invention is intended to encompass fragments of the DNA sequence of the invention in purified form, where the fragments are capable of functioning as promoters for the human villin gene. For example, regulatory elements responsible for tissue specificity of villin can be characterized by a functional test using the Firefly luciferase gene as a reporter gene as described by de Wet et al., *infra*.

The DNA sequence coding for the promoter of the present invention can be amplified in the well known polymerase chain reaction (PCR), which is useful for amplifying all or specific regions of the promoter. See e.g., S. Kwok et al., J. Virol., **61**:1690-1694 (1987); U.S. Patent 4,683,202; and U.S. Patent 4,683,195. More particularly, DNA primers pairs of known sequence positioned 10-300 base pairs apart that are complementary to the plus and minus strands of the DNA to be amplified can be prepared by well known techniques for the synthesis of oligonucleotides. One end of each primer can be extended and modified to create restriction endonuclease sites when the primer is annealed to the DNA. The PCR reaction mixture can contain the DNA, the DNA primer pairs, four deoxyribonucleoside triphosphates, $MgCl_2$, DNA polymerase, and conventional buffers. The DNA can be amplified for a number of cycles. It is generally possible to increase the sensitivity of detection by using a multiplicity of cycles, each cycle consisting of a short period of denaturation of the DNA at an elevated temperature, cooling of the reaction mixture, and polymerization with the DNA polymerase. Amplified sequences can be detected by the use of a technique termed oligomer restriction (OR). See, R. K. Saiki et al., Bio/Technology 3:1008-1012 (1985).

The villin gene is strictly r gulated during embryonic d velopment of th digestiv tract. Villin can be detected in the visceral endoderm, in th primitiv gut, in pancreatic and hepatic anlag , as soon as th s tissues app ar. This expression is then stimulat d in differentiated ent rocytes, r mains low in biliary cells and duct cells, and shut off in hepatocytes and in acinar pancreatic cells. Th DNA s quence encoding th promot r of the human villin g ne is thus us ful to investigate molecular genetics involved in digestive organogenesis. Th villin gene promoter strictly r gulates the expression of villin in a tissu sp cific manner, during adult lif and

during embryonic development.

2. Nucleotide Probes Containing the Gen Promot r

The DNA sequence of the invention coding for the promoter of the human villin gene can also be used as a probe for the detection of a nucleotide sequence in a biological material, such as tissue or body fluids. The probe can be labeled with an atom or inorganic radical, most commonly using a radionuclide, but also perhaps with a heavy metal. Radioactive labels including ^{32}P, ^{3}H, ^{14}C, or the like. Any radioactive label can be employed, which provides for an adequate signal and has sufficient half-life. Other labels include ligands that can serve as a specific binding member to a labeled antibody, fluorescers, chemiluminescers, enzymes, antibodies which can serve as a specific binding pair member for a labeled ligand, and the like. The choice of the label will be governed by the effect of the label on the rate of hybridization and binding of the probe to the DNA or RNA. It will be necessary that the label provide sufficient sensitivity to detect the amount of DNA or RNA available for hybridization.

When the promoter sequence of the invention is used as a probe for hybridizing to a gene, the gene is preferably affixed to a water insoluble solid, porous support, such as nitrocellulose paper. Hybridization can be carried out using labeled polynucleotides of the invention and conventional hybridization reagents. The particular hybridization technique is not essential to the invention.

The amount of labeled probe present in the hybridization solution will vary widely, depending upon the nature of the label, the amount of the labeled probe which can reasonably bind to the support, and the stringency of the hybridization. Generally, substantial excesses of the probe over stoichiometric will be employed to enhance the rate of binding of the probe to the fixed DNA.

Various degrees of stringency of hybridization can be employed. The more severe the conditions, the greater the complementarity that is required for hybridization between the probe and the polynucleotide for duplex formation. Severity can be controlled by temperature, probe concentration, probe length, ionic strength, time, and the like. Conveniently, the stringency of hybridization is varied by changing the polarity of the reactant solution. Temperatures to be employed can be empirically determined or determined from well known formulas developed for this purpose.

3. Nucleotide Sequences Containing the Gene Promoter

This invention also provides the DNA sequence encoding the promoter of the invention linked to nucleic acids, such as nucleic acids comprising a structural gene. The nucleic acid can be obtained from any source, for example, from plasmids, from cloned DNA or RNA, or from natural DNA or RNA from any source, including prokaryotic and eukaryotic organisms. DNA or RNA can be extracted from a biological material, such as biological fluids or tissue, by a variety of techniques including those described by Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1982). The nucleic acid will generally be obtained from a bacteria, yeast, virus, or a higher organism, such as a plant or animal. The nucleic acid can be a fraction of a more complex mixture, such as a portion of a gene contained in whole human DNA or a portion of a nucleic acid sequence of a particular microorganism. The nucleic acid can be a fraction of a larger molecule or the nucleic acid can constitute an entire gene or assembly of genes. The DNA can be in a single-stranded or double-stranded form. If the fragment is in single-stranded form, it can be converted to double-stranded form using DNA polymerase according to conventional techniques.

The DNA sequence coding for the promoter of the human villin gene can be linked to a structural gene. As used herein, the term "structural gene" refers to a DNA sequence that encodes through its template or messenger mRNA a sequence of amino acids characteristic of a specific protein or polypeptide. The nucleotide sequence of the invention can function as an expression control sequence, that is, a DNA sequence that controls and regulates expression of the structural gene when operatively linked to the gene. In one embodiment of this invention, the DNA sequence encoding the human villin gene promoter is operatively linked to the structural gene for human villin. In another embodiment of the invention, the DNA sequence of the invention is operatively linked to a structural gene other than the gene encoding human villin.

The nucleotide sequence of the invention coding for the promoter of the human villin gene can be used in transient assay systems bas d on the us of fusion gen s. Th fusion g n can consist of th promoter of th inv ntion directing th synthesis of a r port r molecul . Th relative amount of th r port r prot in synthesiz d under various conditions is pr sumed to reflect the ability of th promot r to dir ct transcription. Th transi nt expression system should be based on the synthesis of an asily assayed report r protein having minimal or no eff ct on the physiology of th transfected cell. Exampl s of suitabl report r systems ar chloramphenicol acetyltransferase (CAT) and human growth hormone (hGH). Other systems that ar also useful includ th

lucif rase system and ^b-galactosidase, which have b en successfully used as r porter genes in eukaryotic cells. Thus, for example, the DNA s quence of th inv ntion can be operatively linked to a reporter gene, such as the g n ncoding human growth hormon (hGH) or the gen coding for the enzyme luciferase. The Firefly luciferase g ne, its structure, and its expression in mammalian cells ar described in d Wet t al., Mol. Cell. Biol. 7:725-737 (1987).

The DNA sequence encoding the promoter of the human villin gene can also be linked to an oncogene. As used herein, the term "oncogene" means a gene having oncogenic or cancer causing potential. The term as used herein includes cellular oncogenes known as proto-oncogenes, as well as viral oncogenes. When the DNA sequence encoding the promoter for the human villin gene is operatively linked to an oncogene, the resulting polynucleotide is referred to herein as an "activated oncogene sequence."

Previous studies have demonstrated that villin is a marker of digestive epithelium cell lineage. Villin gene expression is strictly regulated during embryogenesis and terminal differentiation. Furthermore, the tissue specific expression of villin is maintained during the carcinogenesis process. Villin can be considered as a marker for colorectal carcinoma and can be used to characterize the colorectal origin of unknown metastasis. As a consequence of these properties, the villin gene is a good candidate to target the expression of oncogenes in the digestive epitheliums of transgenic mice. Oncogenes such as SV40T antigen, whose ability to promote tumors in transgenic mice has been fully demonstrated, K-*ras* or p53, whose mutated forms have been found associated with colorectal cancers in humans, can be placed under the control of the villin promoter of the invention. This makes it possible to obtain transgenic mice developing specific tumors deriving from intestinal epitheliums.

Thus, in a preferred embodiment of the invention, the DNA sequence coding for the promoter of the human villin gene is operatively linked to an oncogene that is associated with tumor cells of the gastrointestinal tract or renal system of the human. Oncogenes associated with colon carcinoma are particularly preferred for use in this invention. Examples of particularly preferred oncogenes are TAG, *ras*, *myc*, and p53 genes. It will be understood, however, that other oncogenes can be linked to the promoter sequence of the invention. For example, the promoter sequence can be linked to oncogenes that have appeared in retroviruses, such as the oncogenes *abl, erb-A, erb-B, ets, fes/fps, fgr, ms, fos, kit, mil/raf, mos, myb, K-ras, rel ros, sis ski, src, yes.* Similarly, the promoter sequence of the invention can be linked to oncogenes that have not appeared in retroviruses, such as the oncogenes *bcl-1, bcl-2, bcr, Blym, dil, int-1,int-2, L-myc, mcf2, mcf3, met, Mlvi-1, Mlvi-2; Mlvi-3 neu N-myc, N-ras, onc-D, pim-1, pvt-1, RMO-int-1, tel-1, Tlym-1, Tlym-2,* T_K*NS-1, tx-1, tx-2, tx-3, tx-4.*

The protein products of many of the oncogenes are protein kinases or protein phosphorylating enzymes. Thus, the activated oncogene sequence can be used to study host expression mechanisms by detecting expression of these products.

4. Vectors Containing the Gene Promoter

This invention also provides cloning and expression vectors containing the DNA sequence of the invention coding for the promoter of the human villin gene.

More particularly, the DNA sequence encoding the promoter can be ligated to a vehicle for cloning the sequence. The major steps involved in gene cloning comprise procedures for separating DNA containing the gene of interest from prokaryotes or eukaryotes, cutting the resulting DNA fragment and the DNA from a cloning vehicle in specific sites, mixing the two DNA fragments together, and ligating the fragments to yield a recombinant DNA molecule. The recombinant molecule can then be transferred into a host cell, and the cells allowed to replicate to produce identical cells containing clones of the original DNA sequence.

The vehicle employed in this invention can be any double-stranded DNA molecule capable of transporting the promoter of the invention into a host cell and capable of replicating within the cell. More particularly, the vehicle must contain at least one DNA sequence that can act as the origin of replication in the host cell. In addition, the vehicle must contain two or more sites for insertion of the DNA sequence encoding the promoter of the invention. These sites will ordinarily correspond to restriction enzyme sites at which cohesive ends can be formed, and which are complementary to the cohesive ends on the promoter sequence be ligated to the vehicle. In general, this invention can be carried out with plasmid, bacteriophage, or cosmid vehicles having these characteristics.

The nucleotid sequence of th invention can hav cohesive ends compatible with any combination of sites in the vehicle. Alternativ ly, th s quenc can have one or more blunt ends that can be ligated to corresponding blunt nds in the cloning sites of th vehicle. The nucleotide sequence to b ligated can b further processed, if desir d, by successive exonuclease d letion, such as with the enzyme Bal 31. In the event that the nucl otide sequ nce of th invention does not contain a desired combination of cohesiv ends, th s quence can b modifi d by adding a linker, an adaptor, or homopolymer tailing.

It is preferred that plasmids used for cloning nucleotide sequences of the invention carry one or more genes responsible for a useful characteristic, such as selectable marker, displayed by the host cell. In a preferred strategy, plasmids having genes for resistance to two different drugs are chosen. For example, insertion of the DNA sequence into a gene for an antibiotic inactivates the gene and destroys drug resistance. The second drug resistance gene is not affected when cells are transformed with the recombinants, and colonies containing the gene of interest can be selected by resistance to the second drug and susceptibility to the first drug. Preferred antibiotic markers are genes imparting chloramphenicol, ampicillin, or tetracyline resistance to the host cell.

A variety of restriction enzymes can be used to cut the vehicle. The identity of the restriction enzyme will generally depend upon the identity of the ends on the DNA sequence to be ligated and the restriction sites in the vehicle. The restriction enzyme is matched to the restriction sites in the vehicle, which in turn is matched to the ends on the nucleic acid fragment being ligated.

The ligation reaction can be set up using well known techniques and conventional reagents. Ligation is carried out with a DNA ligase that catalyzes the formation of phosphodiester bonds between adjacent 5'-phosphate and the free 3'-hydroxy groups in DNA duplexes. The DNA ligase can be derived from a variety of microorganisms. The preferred DNA ligases are enzymes from *E. coli* and bacteriophage T4. T4 DNA ligase can ligate DNA fragments with blunt or sticky ends, such as those generated by restriction enzyme digestion. *E. coli* DNA ligase can be used to catalyze the formation of phosphodiester bonds between the termini of duplex DNA molecules containing cohesive ends.

The vehicle to which the DNA sequence of the invention has been ligated is useful for cloning the sequence. Cloning can be carried in prokaryotic or eukaryotic cells. The host for replicating the cloning vehicle will of course be one that is compatible with the vehicle and in which the vehicle can replicate. When a plasmid is employed, the plasmid can be derived from bacteria or some other organism or the plasmid can be synthetically prepared. The plasmid can replicate independently of the host cell chromosome or an integrative plasmid (episome) can be employed. The plasmid can make use of the DNA replicative enzymes of the host cell in order to replicate or the plasmid can carry genes that code for the enzymes required for plasmid replication. A number of different plasmids can be employed in practicing this invention. Typical of the plasmids that can be utilized are pBR322, pBR325, ColEI, and RP4. A filamentous, lysogenic phage, such as M13 phage, can be employed for cloning the nucleotide sequences of the invention.

The DNA sequence coding for the promoter of the human villin gene and the other nucleotide sequences of the invention, such as the activated oncogene sequence, can also be ligated to a vehicle to form an expression vector. The vehicle employed in this case is one in which it is possible to express a gene operatively linked to the promoter of the invention in an appropriate host cell. It is preferable to employ a vehicle known for use in expressing genes in mammalian cells. These vehicles include SV40 vectors and eukaryotic expression vectors derived from SV40 vectors. Constructs including the DNA sequence encoding the promoter of the invention associated with the gene coding the large T antigen of SV40 are preferred, because the antigen gives transforming power to permissive cells for the expression of the promoter. Other vectors include those derived from Maloney murine sarcoma virus (MSV), herpes simplex virus, Rous sarcoma virus, the mouse mammary tumor virus (MMTV), vaccinia virus, bovine papillomavirus, human adenovirus, and Maloney murine leukemia virus.

In one embodiment of this invention, the DNA sequence coding for the promoter of the human villin gene was operatively linked to the Firefly luciferase gene, and the resulting nucleotide sequence was ligated to plasmid pSVOA using the techniques described in de Wet et al., *supra*. The plasmid pSVOA is shown in Fig. 5.

Referring to Fig. 5, the structures of the mammalian expression vector pSVOA and the full-length, intronless luciferase gene L are shown. The expression vector pSVOA is derived from pSV2, an SV40 early-region promoter vector. Portions of the vector derived from pBR322 are shown as a thin line, those from SV40 are shown as a heavy line. Coordinates of the endpoints of the pBR322 segments are indicated and correspond to the location of the restriction sites in the published sequence of pBR322. Coordinates indicated in bold type correspond to the locations of the restriction sites in the published sequence of SV40. The vector pSVOA contains the SV40 small-t-antigen intron and an SV40 polyadenylation signal labeled A_n. The vector pSVOA contains two copies of the SV40 polyadenylation signal. The full-length, intronless luciferase construct, designated L, is carried as *Hind*III-*Bam*HI fragments in the plasmid vector pUC18. The name of the corresponding plasmid is also indicated below the map. The coordinates of the indicated sites are taken from the sequence shown in Fig. 1 of the de Wet et al. publication, *supra*. Restriction sites are indicated with the following abbreviations: H3, *Hind*III; Ri, *Eco*RI; Bs, *Bsm*I; *Xb*; *Xba*I; H2, *Hinc*II; Sp, *Ssp*I; Sa, *Sac*I; Kp, *Kpn*I; Sm, *Sma*I; Ba, *Bam*HI.

The resulting plasmid derived from pSVOA containing the DNA sequence encoding the promoter of the human villin gene operatively linked to the luciferase gene has been designated pPrVL. The plasmid pPrVL was deposited with the Collection National de Cultures de Microorganisms (C.N.C.M.) of Institut Pasteur in Paris, France on October 26, 1990, under culture collection deposit Accession No. I-1011. The procedure used

to prepar the pPRVL construct is diagrammatically shown in Fig. 6.

Referring to Figure 6, details conc rning the construction of pPrVL are giv n. A HindIII-SalI restriction fragment from pSVOAL containing the luciferase (Luc) g ne was ligated to a 1.7 kb SalI-HindIII fragment obtained from a human genomic library (lambda EMBL 3). The latter fragment was obtain d by scr ening the library using a cocktail of 5′ to 3′ cDNA probes specific for the human villin gene. This construct is referred to as plasmid pPrV 1.7.

A second plasmid was constructed to clone a HindIII/PstI fragment from the same library containing the CAP site of the villin gene. This plasmid was named PrV -300/+77. A HindIII-EcoRI fragment from this plasmid was then ligated to a HindIII fragment from pPrV 1.7 and an EcoRI-HindIII adaptor. The resulting plasmid (pPrVL) contained the 5′ region of the villin gene (1.4 kb insert), the villin promoter and the luciferase gene.

5. Cells Transformed with Vectors Containing the Gene Promoter

The vectors of the invention can be inserted into host organisms using conventional techniques. For example, the vectors can be inserted by transformation, transfection, electroporation, microinjection, or by means of liposomes (lipofection). The preferred method for inserting the vectors into mammalian cells is dependent on cell type; however, calcium phosphate precipitation of DNA has been found to be suitable.

Cloning can be carried in prokaryotic or eukaryotic cells. The host for replicating the cloning vehicle will of course be one that is compatible with the vehicle and in which the vehicle can replicate. Cloning is preferably carried out in bacterial or yeast cells, although cells of fungal, animal, and plant origin can also be employed. The preferred host cells for conducting cloning work are bacterial cells, such as *E. coli*, as well as species of *streptomyces, Bacillus,* and *Pseudomonas*. The use of *E. coli* cells is particularly preferred because most cloning vehicles, such as bacterial plasmids and bacteriophages, replicate in these cells.

In a preferred embodiment of this invention, an expression vector containing the DNA sequence encoding the promoter of the invention operatively linked to a structural gene is inserted into a mammalian cell using conventional techniques. For example, the vector can be transfected into a mammalian cell by the calcium phosphate precipitation method or the DEAE-dextran method. A preferred method based on calcium phosphate precipiation is disclosed in Chen and Olsyama, Mol. Cell. Biol., 7:2745-2752 (1987).

Suitable host cells include cells that express villin and cells that do not express villin. Host cells that are preferred for this purpose include human intestinal cell lines derived from colic adenocarcinoma, such as HT29 or CaCO2 cells; human epithelial cell lines, such as C33 cells; and human hepatoma cell lines, such as HepG2 cells; cell lines originating from the renal, proximal tubule of a pig, such as LLCPK1; and cells that do not express villin, such as HeLa cells, which originate from an adecarcinoma of the neck of the uterus. After the plasmid has been introduced into the host cell, the structural gene can be expressed using conventional techniques. These cell lines can be used with recombinant vectors containing the Firefly luciferase gene under the control of the DNA sequence encoding the promoter of the invention in order to isolate regions of the promoter that can be employed for the specific expression of the villin gene in a limited number of tissues. Cell lines have been transfected with plasmid pPRVL of the invention. This plasmid contains the Firefly luciferase reporter gene.

6. Transgenic, Non-Human Mammals

Normal mice do not develop intestinal tumors spontaneously. It is possible, however, to induce adenocarcinomas from small and large intestines by using chemical agents. The histology of these tumors resembles that of corresponding human tumors, and they have been used to study external factors involved in the carcinogenesis process. The usefulness of this model is, however, limited for two main reasons. First of all, the model reproduces only some aspects of human carcinogenesis. For instance, metastasis are not observed, especially in the liver. Secondly, tumors have to be induced chemically in each mouse to be studied. This reduces the reproducibility of the model. Moreover, tumor development is very slow (average 7 months).

Transgenic mouse technology is very suitable to develop animal models of carcinogenesis, since this technology makes it possible to obtain mouse lines that genetically develop specific cancers in a reproducible manner, even those that do not appear spontaneously in this animal.

Accordingly, this invention also provid s transgenic animals containing the promot r sequence of th invention. More particularly, the invention provides a transgenic non-human mammal whose g rm cells and somatic cells contain an activated oncogene sequence of the invention introduced into the animal, or an ancestor of the animal (e.g. an F1 animal), at an embryonic stage. Preferably, th non-human mammal is a rodent, such as a mouse. The activated oncogene sequenc , when incorporated into the genome of the mammal, increases the probability of the d velopment of neoplasms, particularly malignant tumors, in th animal. In a

preferred embodiment of th invention, the neoplasms are analogs of human colon cancer.

Th r are several methods by which the oncogene s quence of th inv ntion can be introduced into an animal mbryo to ensure that it is incorporated into the chromosome of the animal in an activated stat . On method is to transfect the embryo with the g ne and to select transgenic animals in which the gen has integrated into the chromosome at a locus that results in activation. Other methods involve modifying the oncogene prior to introduction into the embryo. One such method is to transfect the embryo using a vector containing the activated oncogene sequence of the invention. In a preferred embodiment of the invention, the chromosome of the transgenic animal includes an endogenous coding sequence, preferably the p53, *ras*, *myc*, or Tag gene, which is substantially the same as the oncogene sequence, and transcription of the oncogene sequence is under the control of the promoter sequence of the invention.

Introduction of the activated oncogene sequence of the invention at the fertilized oocyte stage, such as by microinjection, ensures that the oncogene sequence will be present in all of the germ cells and somatic cells of the transgenic animal, such as the mouse. The presence of the activated oncogene sequence in the germ cells of the transgenic animal ensures that all of the descendants of the animal will carry the activated oncogene sequence in all of their germ cells and somatic cells. The activated oncogene sequence of the invention can be introduced at a later embryonic stage, although this might result in the absence of the oncogene from some somatic cells of the parent animal, although descendants of the animal that inherit the oncogene sequence will carry the sequence in all of their germ cells and somatic cells.

The animals of the invention can be used to test a material suspected of being a carcinogen by exposing the animal to the material and determining neoplastic growth as an indicator of carcinogenicity. The animals can also be used to test materials for ability to confer protection against the development of neoplasms. In either case, the animals are treated with the material, and a reduced incidence of neoplasm development compared to untreated animals is detected as an indication of carcinogenicity or protection. The invention also provides methods of exposing treated and untreated animals to carcinogen prior to, after, or simultaneously with treatment with a protective material.

The transgenic animal model of the invention provides an experimental approach to the different early and late steps of carcinogenesis, and in particular for studying oncogene implication and synergy, the apparition of proliferative hyperplasy, which occurs before the development of solid tumors, and the correlation with angiogenesis.

The villin promoter of the invention is the only promoter isolated to date, which is able to drive the expression of an oncogene in the epithelium of the large intestine. Thus, the DNA sequence encoding the promoter of the human villin gene is the only way to obtain transgenic animals developing colorectal carcinogenesis.

7. Cultures of Transgenic Animal Cells

Intestinal cell lines derived from spontaneous colorectal carcinomas are available. These cell lines have been used to establish cell culture models for absorptive enterocytes and goblet cells. However, the establishment of cell lines for PANETH entero-endocrine cells, and M cells has failed. Furthermore, no well defined cell line models for duct cells of the exocrine pancreas and for biliary cells of the liver are available.

The transgenic animals of the invention can be used as a source of cells for cell culture. Cells from the transgenic animals advantageously exhibit desirable properties of both normal and transformed cultured cells. That is, they are normal or nearly normal morphologically and physiologically, but can be cultured for longer, and even indefinite, periods of time.

More particularly, tissues of the transgenic animals of the invention can be cultured in order to obtain stable cell lines, which reproduce the differentiated phenotype of the corresponding epithelium, and which can be analyzed for the presence of the activated oncogene, either by directly analyzing DNA or RNA, or by assaying the tissue for protein expressed by the gene. Cells of tissues carrying the gene can be cultured using standard tissue culture techniques. The cells are useful for studying the functioning of cells from normally difficult to culture tissues. The cells can be used to study terminal differentiation processes. More particularly, cultures of the transgenic animal cells can be used to characterize the epithelial lines that possess specific differentiation criteria, either of the small intestine or the colon, of ductal cells of the pancreas, or of biliary cells of the liver or of the kidney proximal tubule.

Fig. 7 is a diagrammatic representation of th preparation of an animal cell mod l for colorectal cancers and cell cultures derived from animal tumors.

Referring to Figure 7, details are given concerning the protocol which is followed to obtain th animal and cell cultur mod l for colorectal cancers. A vector containing th villin promoter and an oncogen (preferably p53, *ras*, *myc* or *Tag*) is transfected into a mouse oocyte. A selection procedure is used to establish which mice carry the desired gen s. Thes animals ar maintained as a colony in which the g nes can b readily detected

in both somatic and germ cells.

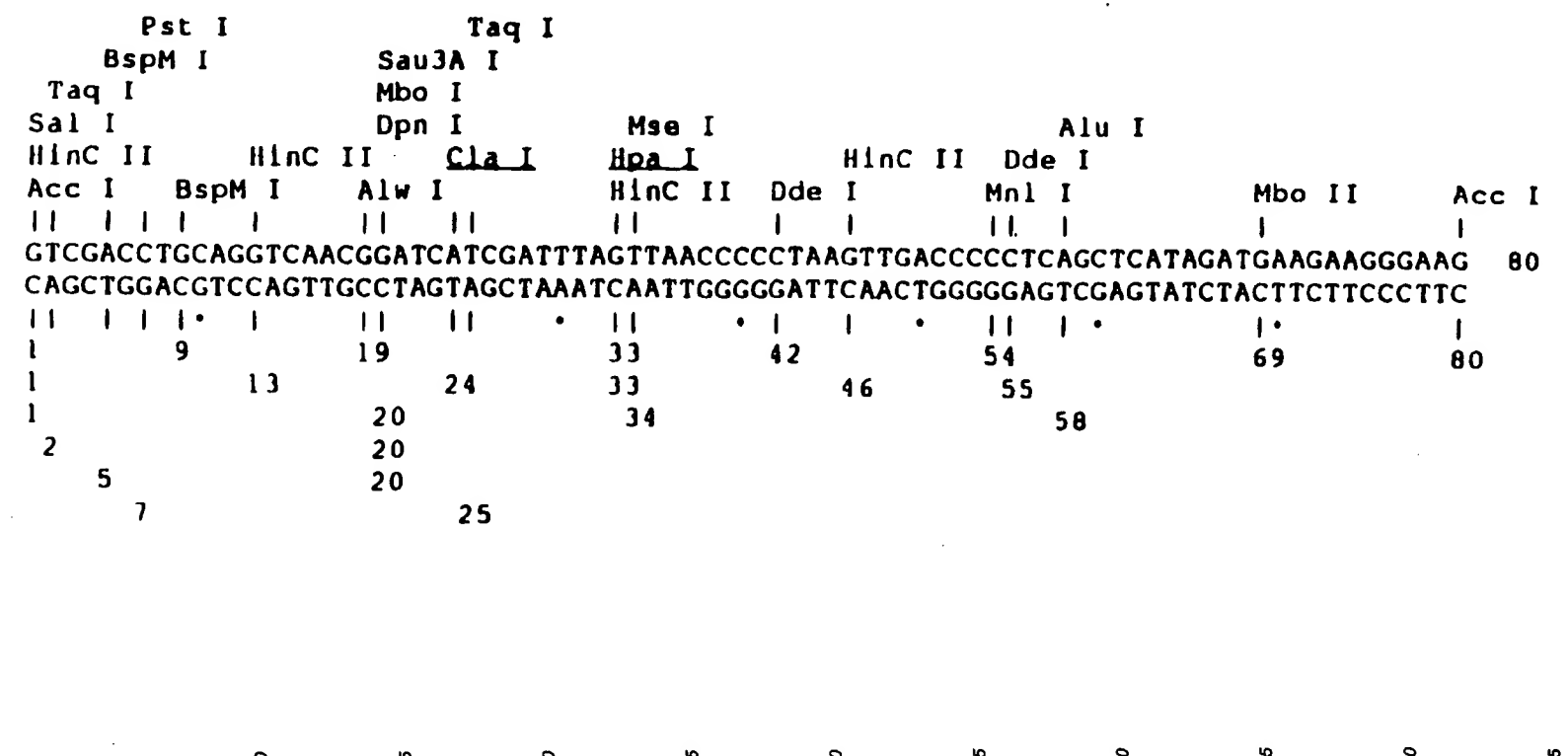
Animals carrying th activated oncog ne can be then be used to investigate, for xampl , the induction of tumors in the digestive epithelium where villin is normally xpressed. Using these animals one can develop an animal model for colorectal canc rs. Alternatively, one can establish cell lines in culture from the digestive epithelium of transgenic animals carrying the villin gene and promoter which might be coupled to *ras* or *myc* to permit the *in vitro* study of colorectal tumor cells.

Claims

1/ An isolated DNA sequence corresponding to a promoter of the human villin gene, wherein the nucleotide sequence consists essentially of :

PRV.STRIDER -> Restriction Map

DNA sequence 1991 b.p. GTCGACCTGCAG ... CTGCAGGTCGAC linear



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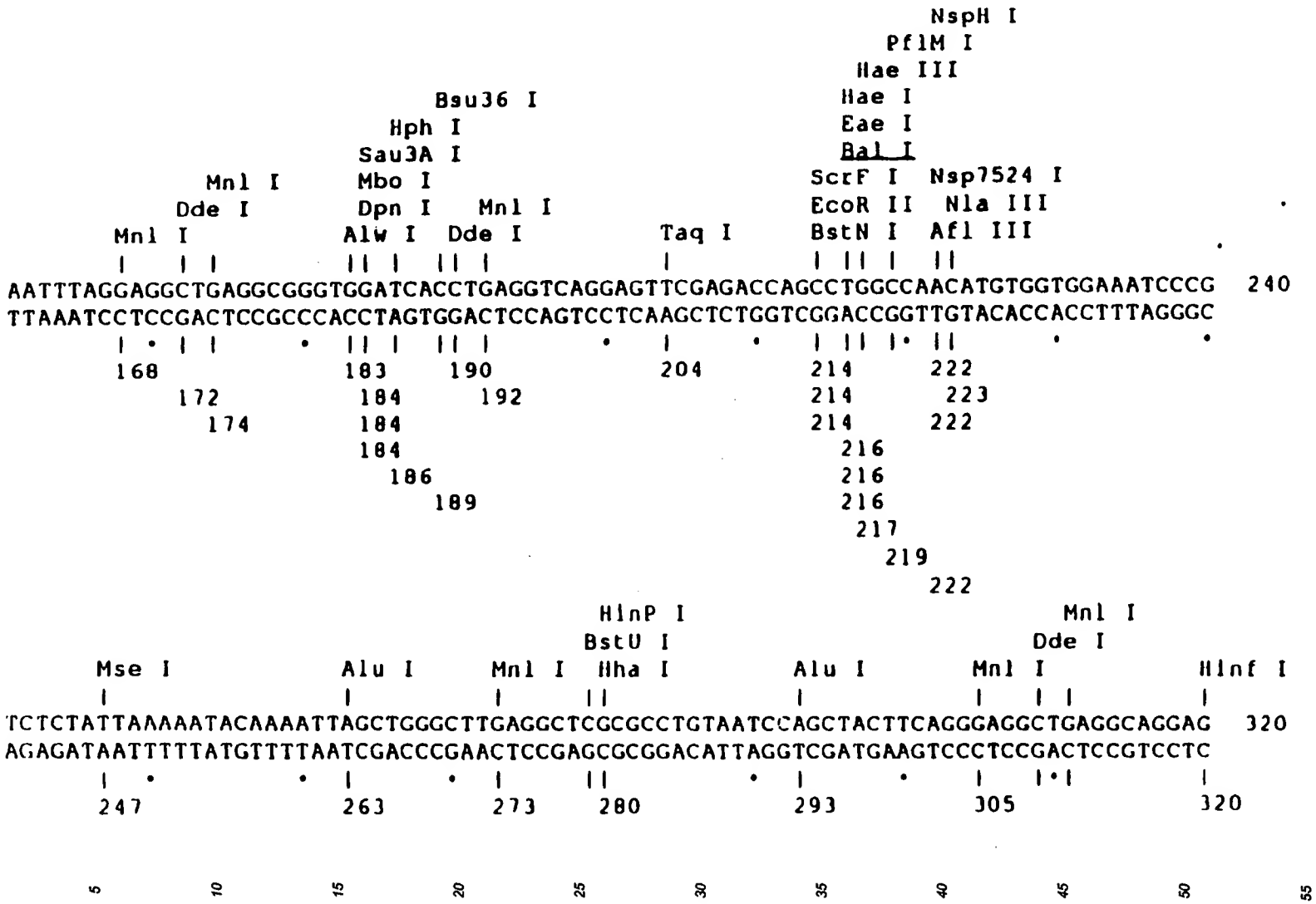
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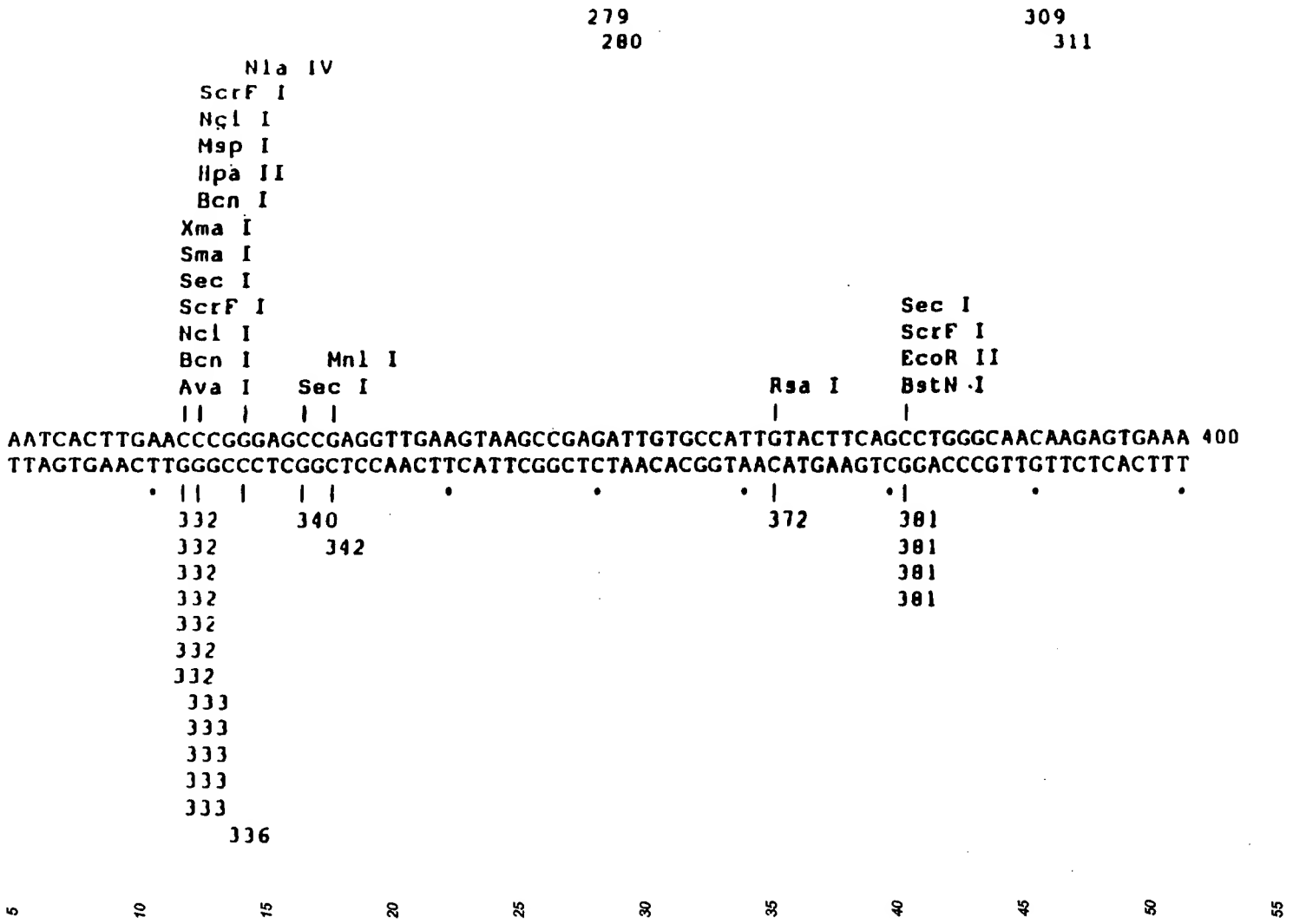
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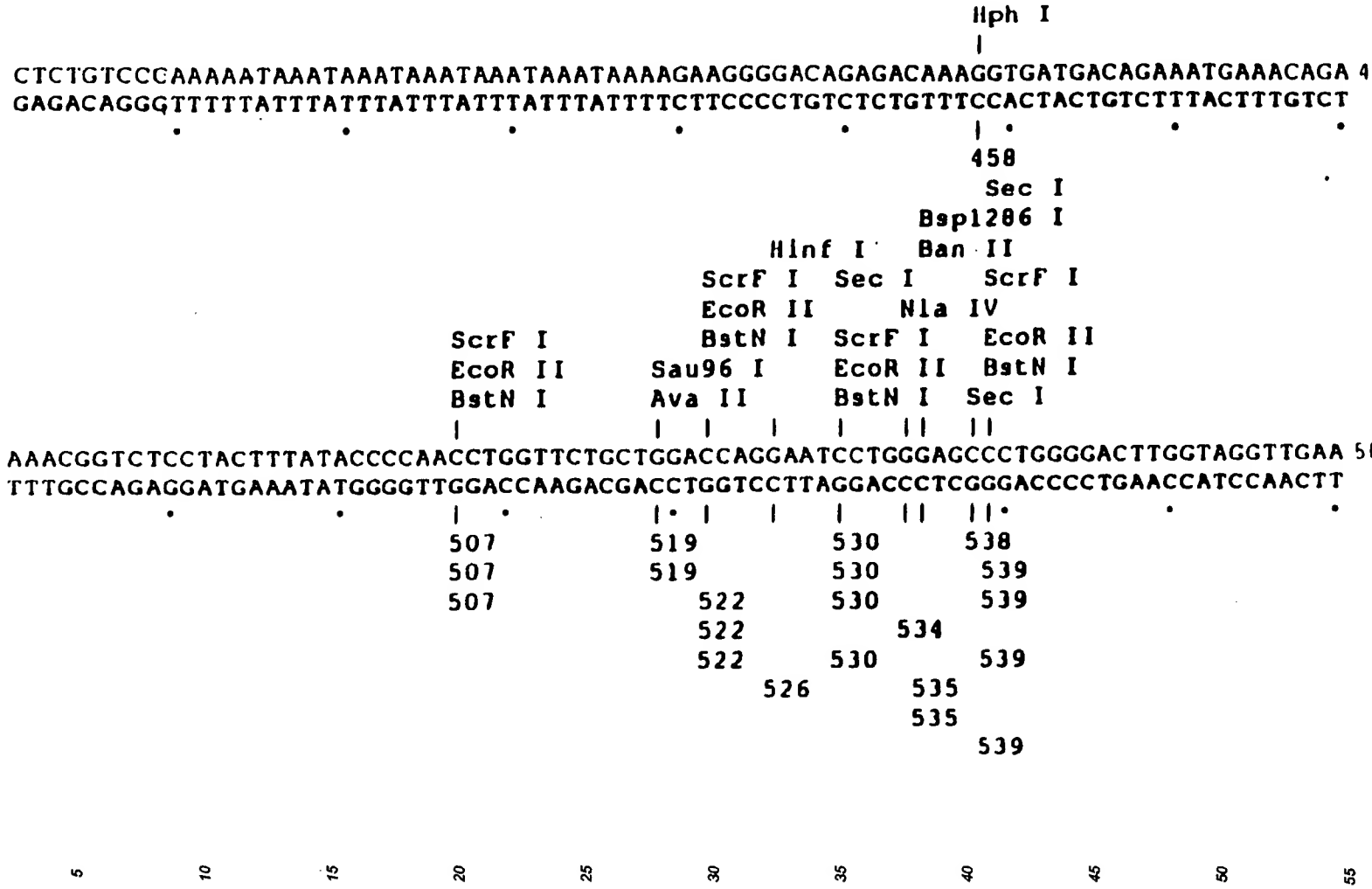
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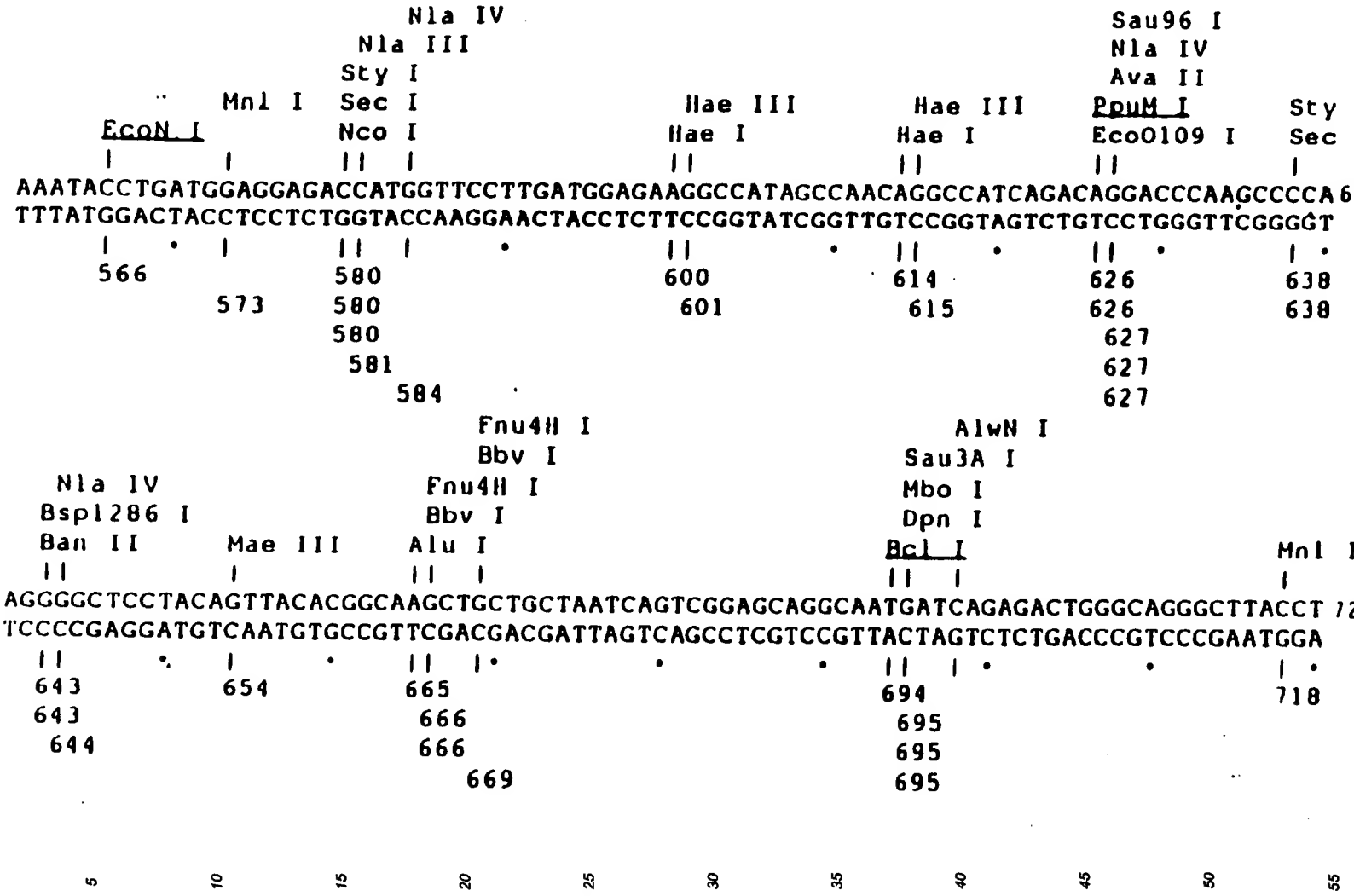
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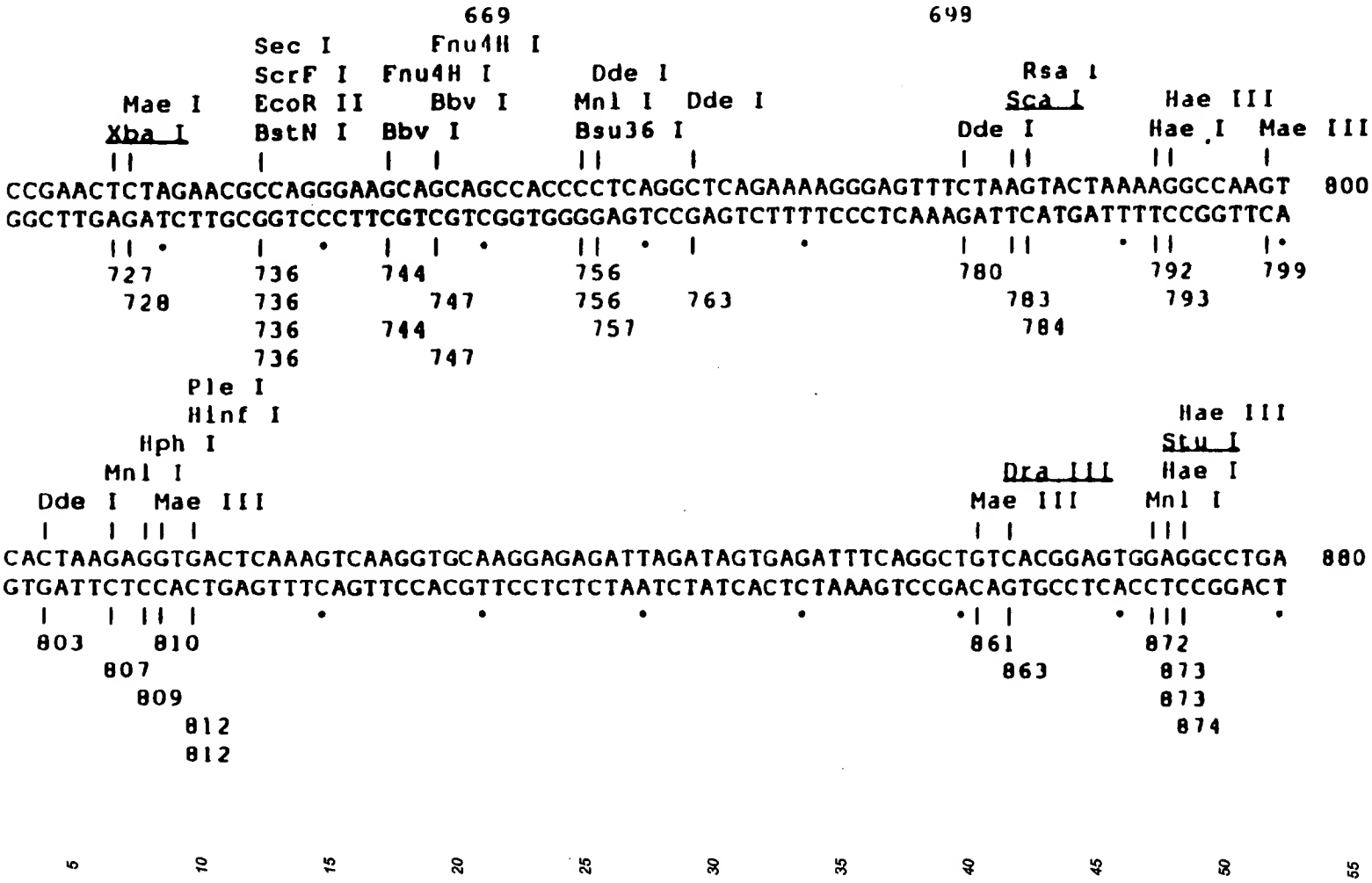
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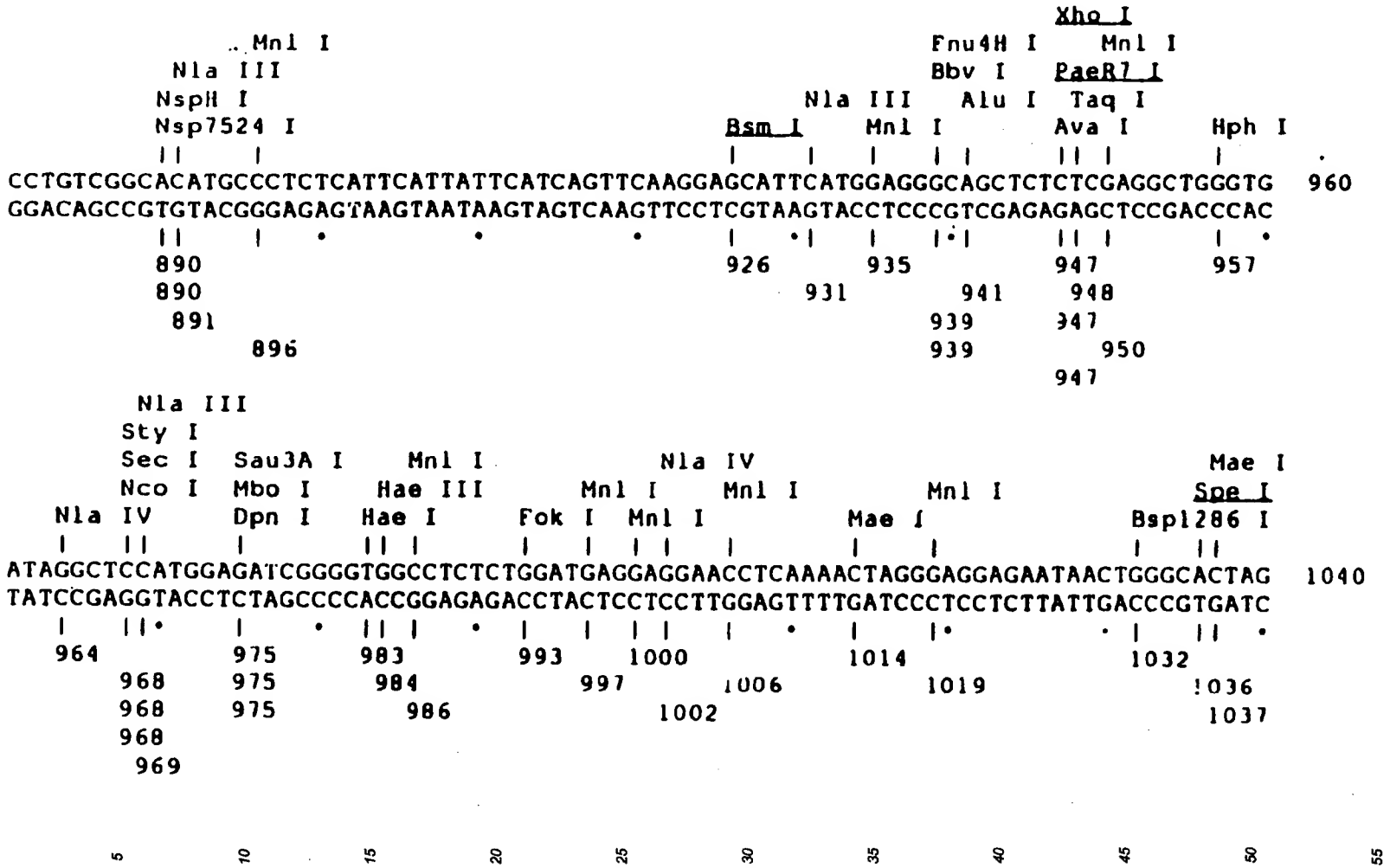
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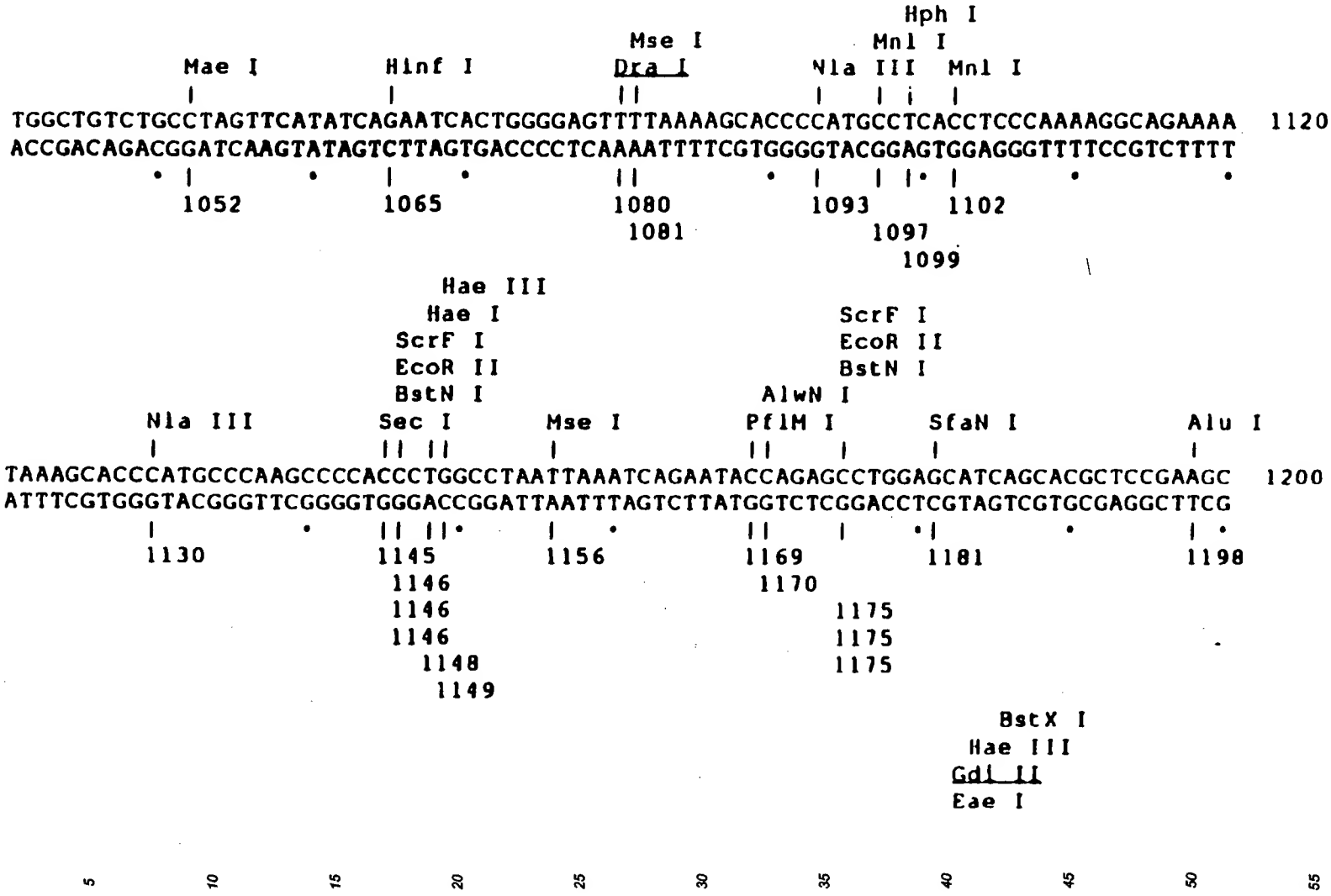
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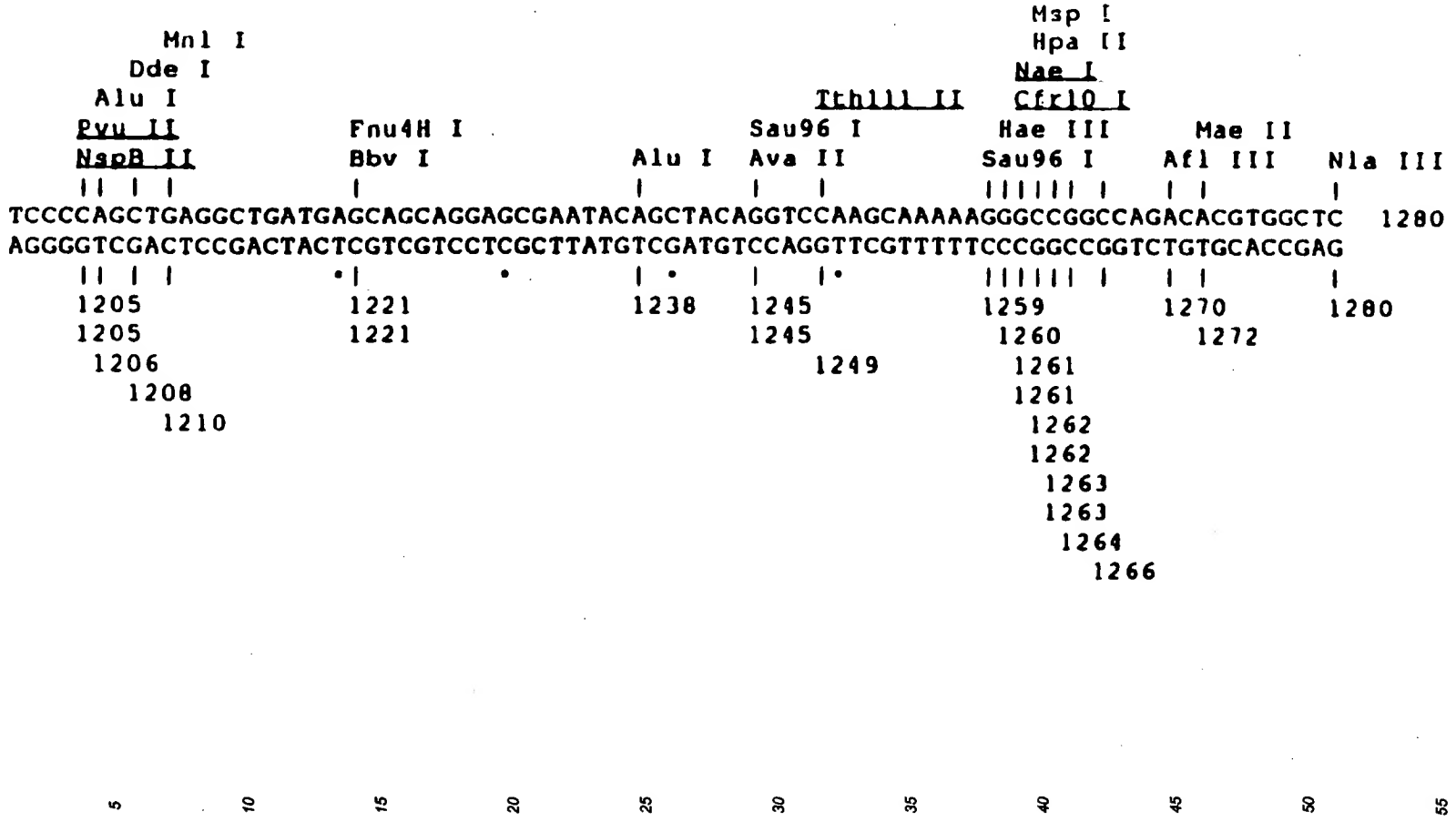
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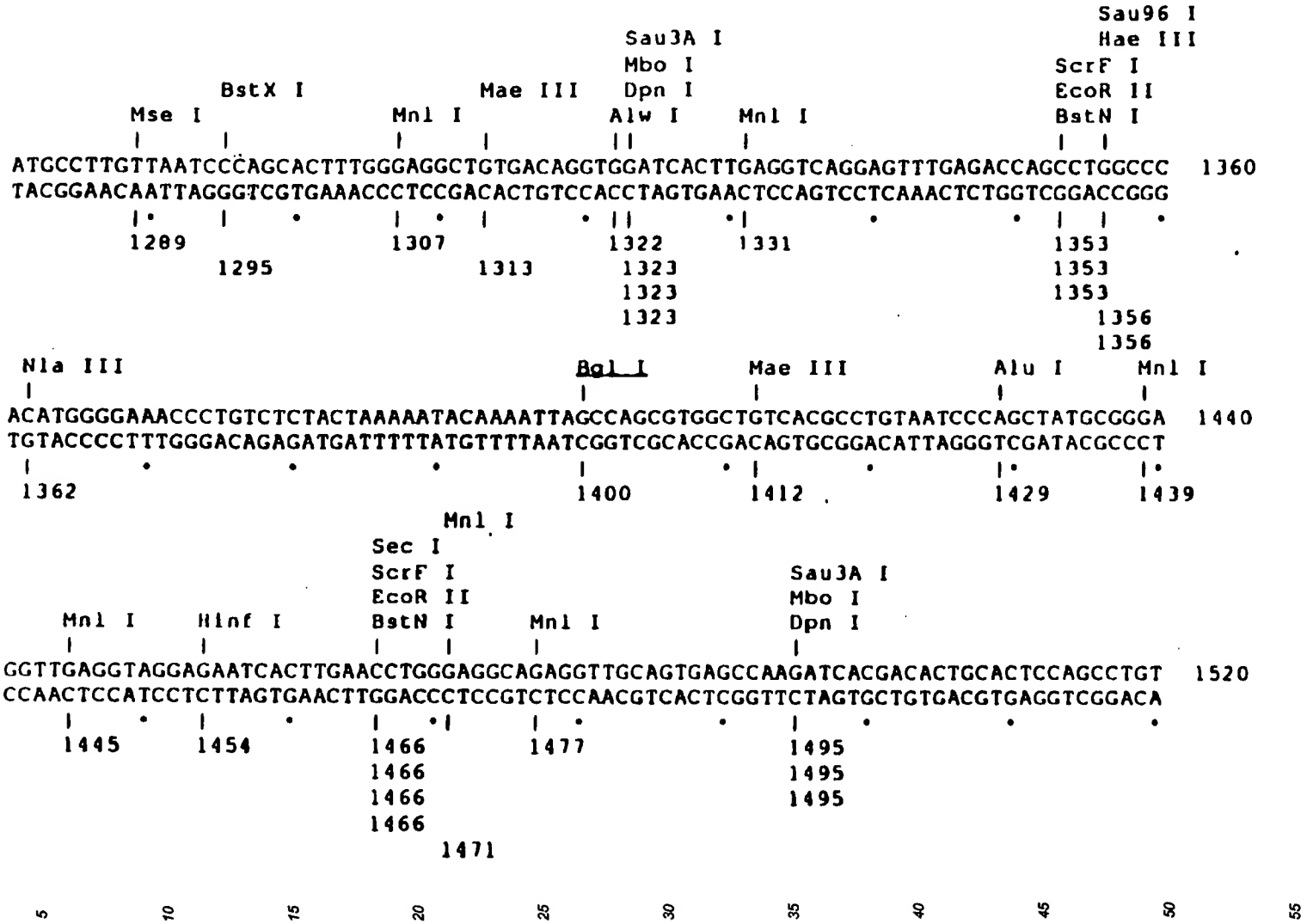
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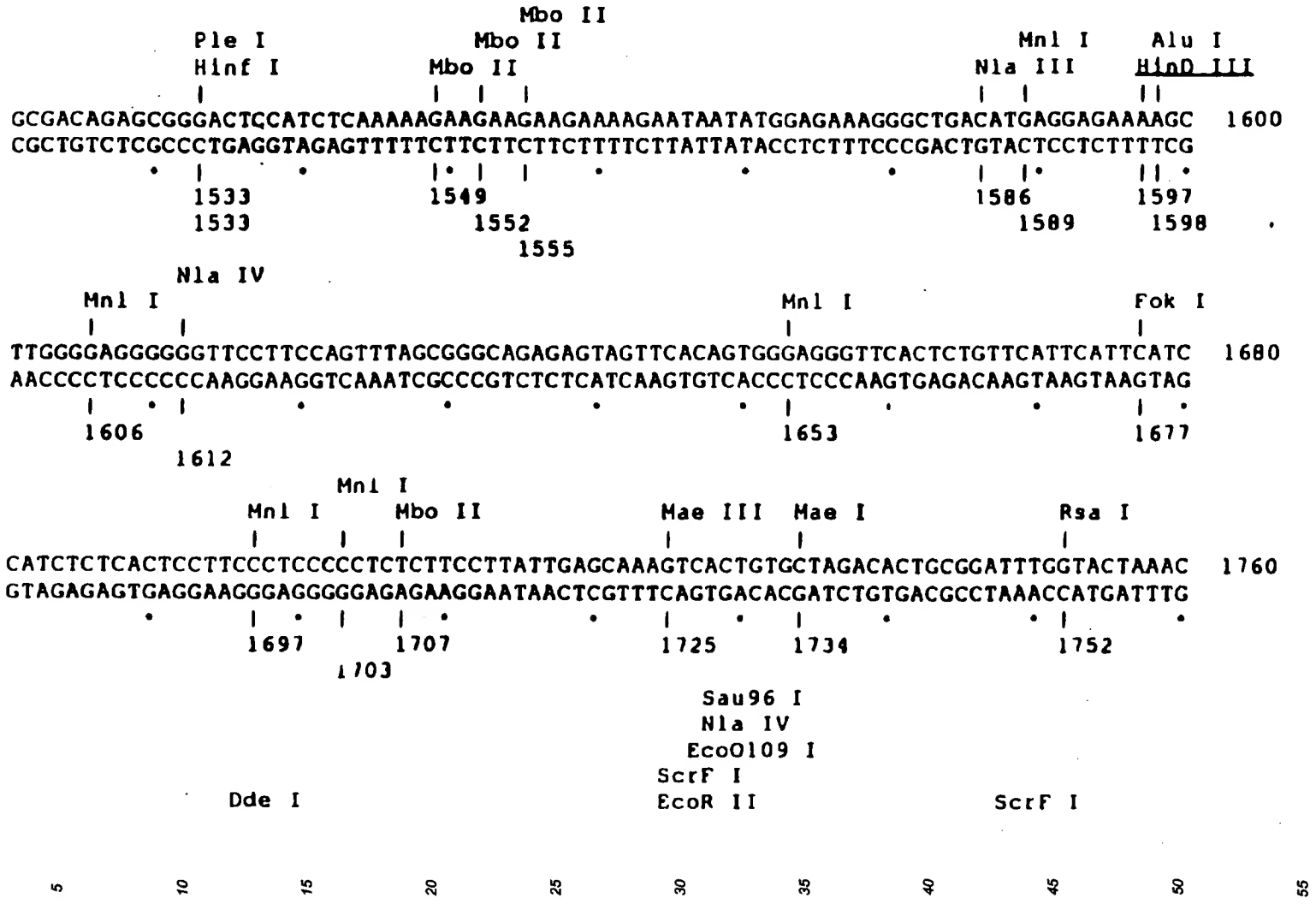
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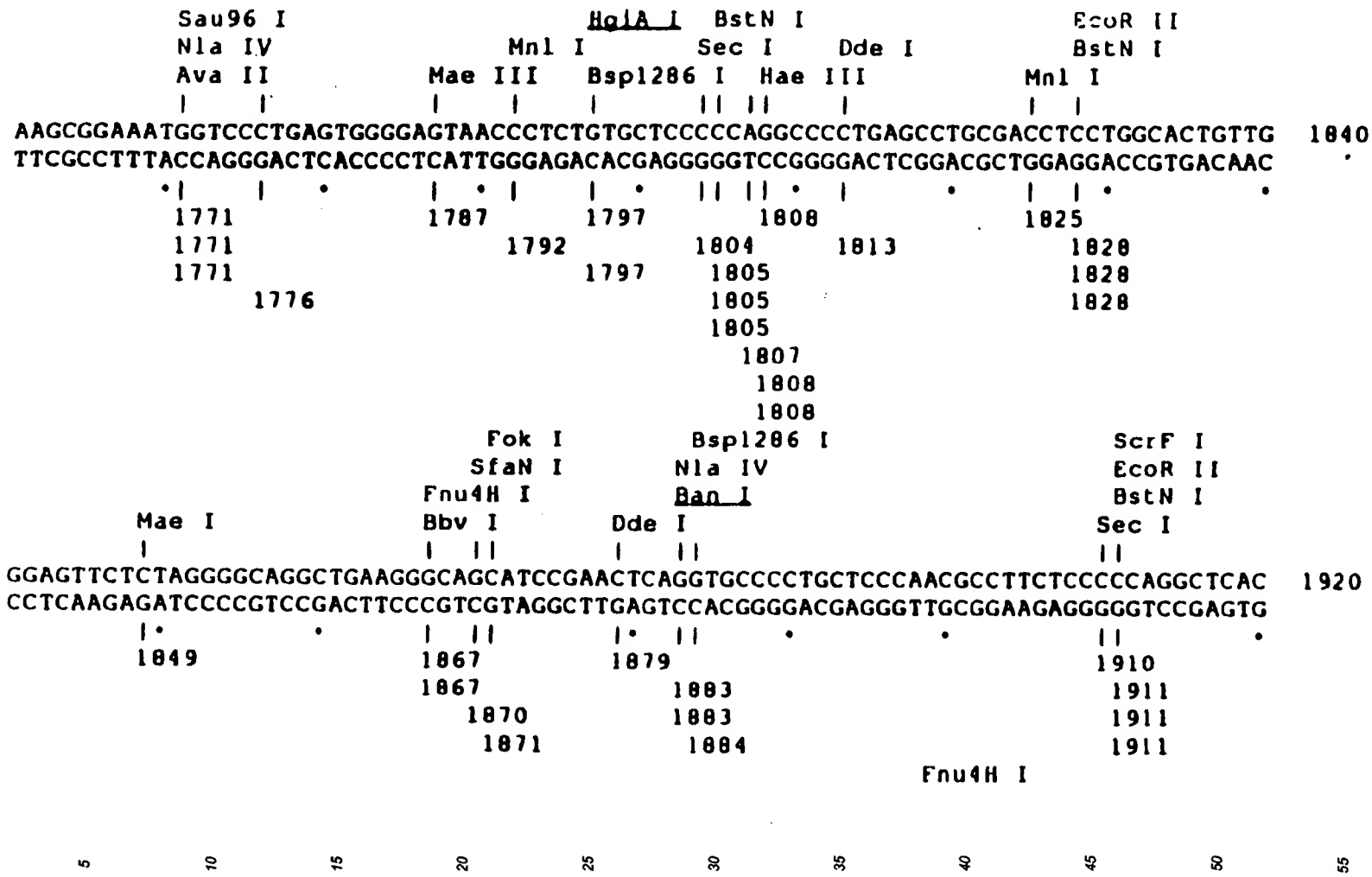
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1808
1813
1825
1828
1828
1828
Fok I
SfaN I
Fnu4H I
Bbv I
Bsp1286 I
Nla IV
Ban I
Dde I
ScrF I
EcoR II
BstN I
Sec I
Mae I
GGAGTTCTCTAGGGGCAGGCTGAAGGGCAGCATCCGAACTCAGGTGCCCCTGCTCCCAACGCCTTCTCCCCCAGGCTCAC 1920
CCTCAAGAGATCCCCGTCCGACTTCCCGTCGTAGGCTTGAGTCCACGGGGACGAGGGTTGCGGAAGAGGGGGTCCGAGTG
1849
1867
1867
1870
1871
1879
1883
1883
1884
1910
1911
1911
1911
Fnu4H I
5
10
15
20
25
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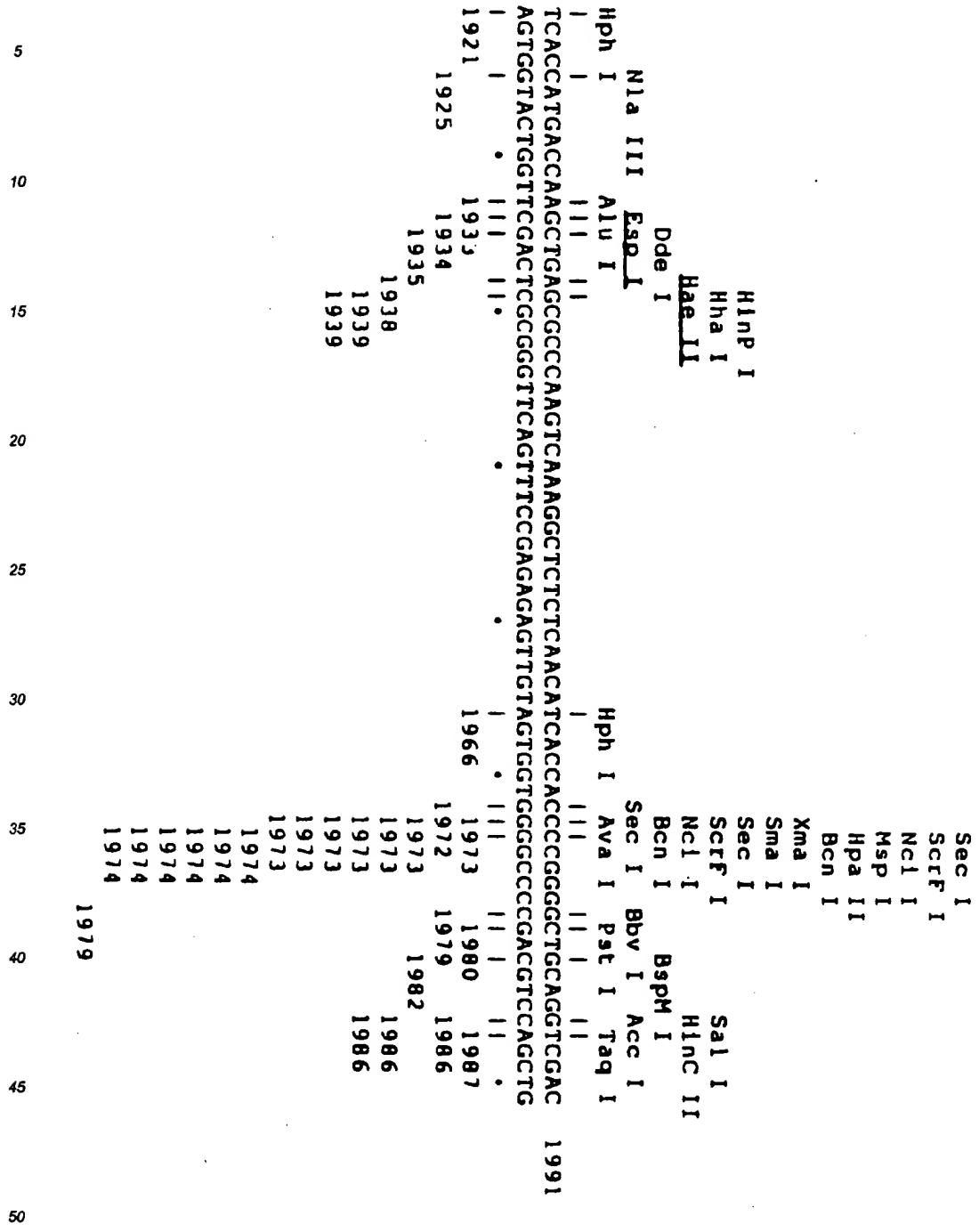
40
45
50
55



2/ A DNA sequence comprising the sequence as claimed in claim 1 operatively linked to a nucleotide sequence of an oncogene.

3/ Th DNA sequence as claimed in claim 1 or 2, wherein the oncogene is selected from th group consisting of p53, ras, myc and Tag.

4/ An isolated DNA sequence as claimed in claim 1 operatively link d to a structural gene.

5/ An isolated DNA sequence comprising the sequ nce as claimed in claim 1 or 4 op ratively linked to a

gene encoding villin.

6/ A DNA sequence comprising the sequence as claimed in claim 1 operatively linked to a gene encoding luciferase.

7/ An isolated RNA sequ nc complementary to th nucleotide sequ nce according to anyone of claims 1 to 6.

8/ A vehicle comprising a plasmid a plasmid or viral vector containing the nucleotide sequence as claimed in anyone of claims 1 to 6.

9/ The vehicle as claimed in claim 8, wherein the viral vector is an SV-40 vector.

10/ The vehicle as claimed in claim 8, wherein the plasmid is pSVOAL.

11/ The vehicle as claimed in claim 8 having the identifying characteristics of the vector having culture collection accession number CNCM (Collection Nationale des Microorganismes, Paris, France) I-1011.

12/ The vehicle as claimed in claim 8, wherein the vector is an expression vector.

13/ A transgenic, non-human mammal all of whose germ cells and somatic cells contain a recombinant activated oncogene sequence according to anyone of claims 2 to 5, 5, which has been introduced into the mammal, or an ancestor of the mammal, at an embryonic stage.

14/ The mammal as claimed in claim 13, wherein a chromosome of the mammal includes an endogenous coding sequence substantially the same as a coding sequence of said oncogene sequence.

15/ The mammal as claimed in claim 14, wherein the oncogene sequence has been integrated into a chromosome of the mammal at a site different from the location of the endogenous coding sequence.

16/ The mammal as claimed in anyone of claims 13 to 15, wherein the mammal is a rodent.

17/ The animal as claimed in anyone of claims 13 to 15, wherein the mammal is a mouse.

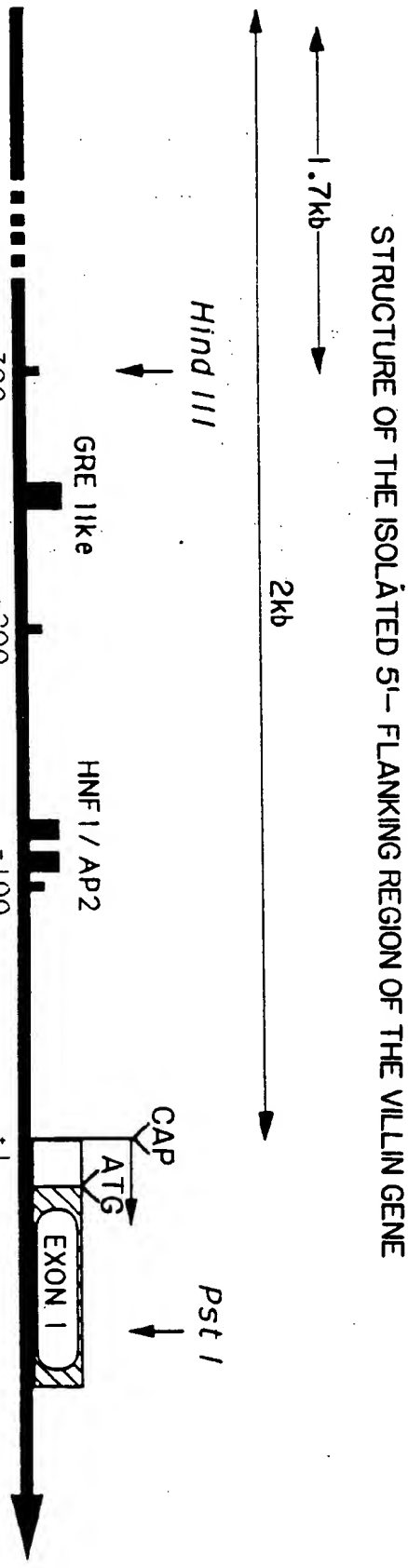

STRUCTURE OF THE ISOLATED 5'- FLANKING REGION OF THE VILLIN GENE
1.7kb
2kb
Hind III
Pst I
-300
-200
-100
+1
GRE like
HNF 1 / AP2
CAP
ATG
EXON 1

FIG. 1'

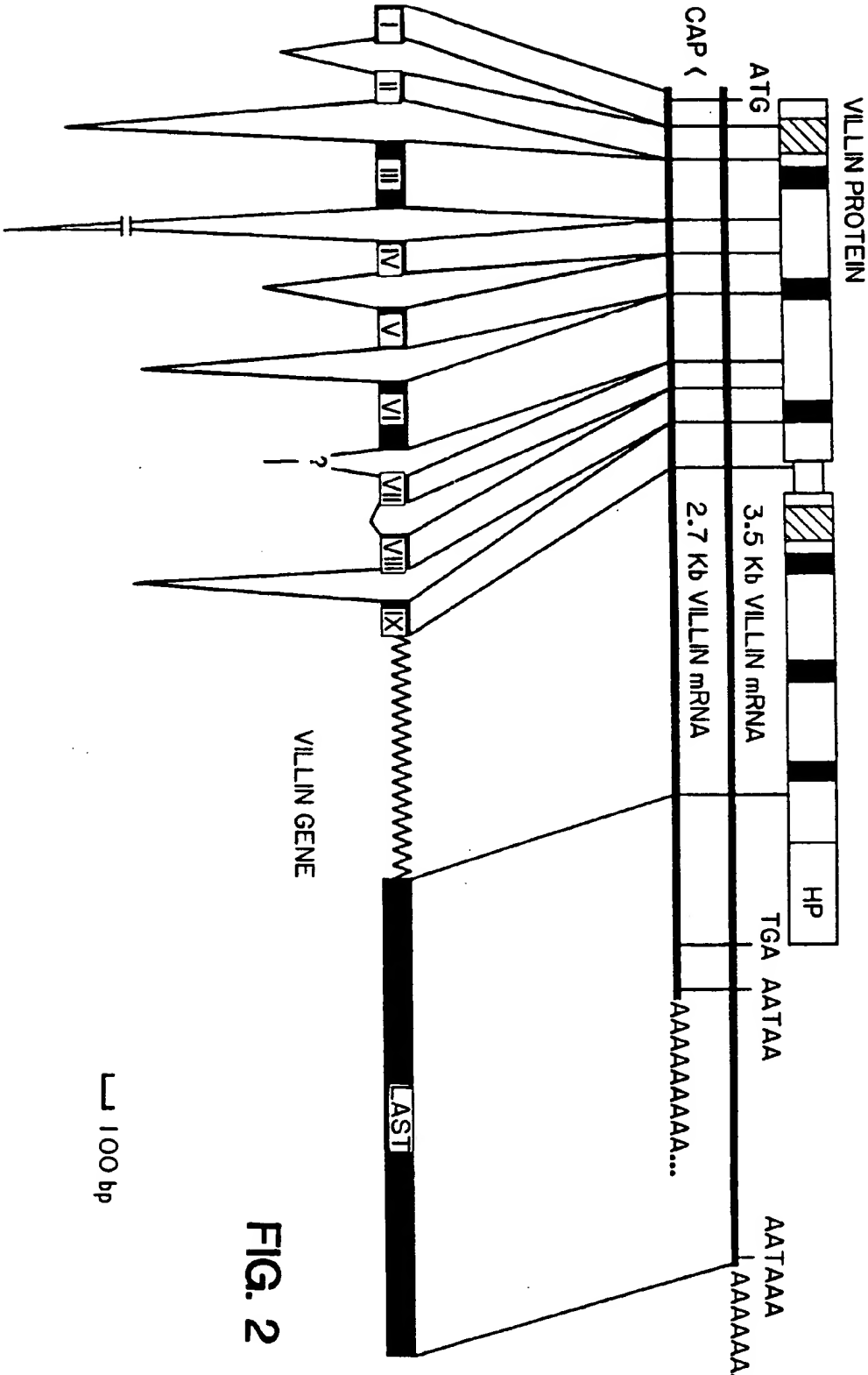

VILLIN PROTEIN
CAP
ATG
I
II
III
IV
V
VI
?
VII
VIII
IX
3.5 Kb VILLIN mRNA
2.7 Kb VILLIN mRNA
HP
TGA
AATAA
AAAAAAAAA...
AATAAA
AAAAAAAAA...
VILLIN GENE
LAST
100 bp

FIG. 2

Cap site */Met.....*

CCTCTCCCCCAGGCTCACTCACC ATG ACC AAG CTG AGC GCC CAA GTC AAA GGC TCT CTC AAC ATC ACC ACC CCG GGG CTG CAG ATA TGG AGG ATC GAG*(+97)* --- ***IVS 1 (390 bp)*** --- GCC ATG CAG ATG GTG CCT GTT CCT TCC AGC ACC TTT GGA AGC TTC TTC GAT GGT GAC TGC TAC ATC ATC CTG GCT*(+172)* --- ***IVS 2 (1300 bp)*** --- ATC CAC AAG ACA GCC AGC AGC CTG TCC TAT GAC ATC CAC TAC TGG ATT GGC CAG GAC TCA TCC CTG GAT GAG CAG GGG GCA GCT GCC ATC TAC ACC ACA CAG ATG GAT GAC TTC CTG AAG GGC CGG GCT GTG CAG CAC CGC GAG GTC CAG GGC AAC GAG AGC GAG GCC TTC CGA GGC TAC TTC AAG CAA GGC CTT GTG*(+370)* --- ***IVS 3 (>2000 bp)*** --- ATC CGG AAA GGG GGC GTG GCT TCT GGC ATG AAG CAC GTG GAG ACC AAC TCC TAT GAC GTC CAG AGG CTG CTG CAT GTC AAG GGC AAG AGG AAC GTG GTA GCT GGA G*(+476)* --- ***IVS 4 (450 bp)*** --- AG GTA GAG ATG TCC TGG AAG AGT TTC AAC CGA GGG GAT GTT TTC CTC CTG GAC CTT GGG AAG CTT ATC ATC CAG TGG AAT GGA CCG GAA AGC ACC CGT ATG GAG AGA CTC AGG G*(+590)* --- ***IVS 5 (1000 bp)*** --- GC ATG ACT CTG GCC AAG GAG ATC CGA GAC CAG GAG CGG GGA GGG CGC ACC TAT GTA GGC GTG GTG GAC GGA GAG AAT GAA TTG GCA TCC CCG AAG CTG ATG GAG GTG ATG AAC CAC GTG CTG GGC AAG CGC AGG GAG CTG AAG GCG GCC GTG CCC GAC ACG GTG GTG GAG CCG GCA CTC AAG GCT GCA CTC AAA CTG TAC CA*(+792)* --- ***IVS 6*** --- T GTG TCT GAC TCC GAG GGG AAT CTG GTG GTG AGG GAA GTC GCC ACA CGG CCA CTG ACA CAG GAC CTG CTC AGT CAC GAG GA*(+873)* --- ***IVS 7*** --- C TGT TAC ATC .....

FIG. 3

*GTCGACCTGCAGGTCAACG*G ATCATCGATTTAGTTAACCC CCTAAGTTGACCCCCTCAGC - 1850

TCATAGATGAAGAAGGGAAG TCTACGTTCATACAGCTAGT GAGTGTCAAAGAAAAAGAGG - 1790

GACGGGGCCCGGCGCGGTGG CTCACGCCTGTAATTCCAGC AATTTAGGAGGCTGAGGCGG - 1730

GTGGATCACCTGAGGTCAGG AGTTCGAGACCAGCCTGGCC AACATGTGGTGGAAATCCCG - 1670

TCTCTATTAAAAATACAAAA TTAGCTGGGCTTGAGGCTCG CGCCTGTAATCCAGCTACTT - 1610

CAGGGAGGCTGAGGCAGGAG AATCACTTGAACCCGGGAGC CGAGGTTGAAGTAAGCCGAG - 1550

ATTGTGCCATTGTACTTCAG CCTGGGCAACAAGAGTGAAA CTCTGTCCCAAAAATAAATA - 1490

AATAAATAAATAAATAAAAG AAGGGGACAGAGACAAAGGT GATGACAGAAATGAAACAGA - 1430

AAACGGTCTCCTACTTTATA CCCCAACCTGGTTCTGCTGG ACCAGGAATCCTGGGAGCCC - 1370

TGGGGACTTGGTAGGTTGAA AAATACCTGATGGAGGAGAC CATGGTTXXXXCCTTGATGG - 1310

AGAAGGCCATAGCCAACAGG CCATCAGACAGGACCCAAGC CCCAAGGGGCTCCTACAGTT - 1250

ACACGGCAAGCTGCTGCTAA TCAGTCGGAGCAGGCAATGA TCAGAGACTGGGCAGGGCTT - 1190

ACCTCCGAACTCTAGAACGC CAGGGAAGCAGCAGCCACCC CTCAGGCTCAGAAAAGGGAG - 1130

TTTCTAAGTACTAAAAGGCC AAGTCACTAAGAGGTGACTC AAAGTCAAGGTGCAAGGAGA - 1070

GATTAGATAGTGAGATTTCA GGCTGTCACGGAGTGGAGGC CTGACCTGTCGGCACATGCC - 1010

CTCTCATTCATTATTCATCA GTTCAAGGAGCATTCATGGA GGGCAGCTCTCTCGAGGCTG - 950

GGTGATAGGCTCCATGGAGA TCGGGGTGGCCTCTCTGGAT GAGGAGGAACCTCAAAACTA - 890

GGGAGGAGAATAACTGGGCA CTAGTGGCTGTCTGCCTAGT TCATATCAGAATCACTGGGG - 830

AGTTTTAAAAGCACCCCATG CCTCACCTCCCAAAAGGCAG AAAATAAAGCACCCATGCCC - 770

AAGCCCCACCCTGGCCTAAT TAAATCAGAATACCAGAGCC TGGAGCATCAGCACGCTXCC - 710

GAAGCTCCCCAGCTGAGGCT GATGAGCAGCAGGAGCGAAT ACAGCTACAGGTCCAAGCAA - 650

AAAGGGCCGGCCAGACACGT GGCTCATGCCTTGTTAATCC CAGCACTTTGGGAGGCTGTG - 590

ACAGGTGGATCACTTGAGGT CAGGAGTTTGAGACCAGCCT GGCCCACATGGGGAAACCCT - 530

GTCTCTACTAAAAATACAAA ATTAGCCAGCGTGGCTGTCA CGCCTGTAATCCCAGCTATG - 470

CGGGAGGTTGAGGTAGGAGA ATCACTTGAACCTGGGAGGC AGAGGTTGCAGTGAGCCAAG - 410

FIG. 4'A

ATCACGACACTGCACTCCAG CCTGTGCGACAGAGCGGGAC TCCATCTCAAAAAGAAGAAG - 350

AAGAAAAGAATAATATGGAG AAAGGGCTGACATGAGGAGA AAAGCTTGGGGAGGGGGGTT - 290

CCTTCCAGTTTAGCGGGCAG AGAGTAGTTCACAGTGGGAG GGTTCACTCTGTTCATTCAT - 230

TCATCCATCTCTCACTCCTT CCCTCCCCCTCTCTTCCTTA TTGAGCAAAGTCACTGTGCT - 170

AGACACTGCGGATTTGGTAC TAAACAAGCGGAAATGGTCC CTGAGTGGGGAGTAACCCTC - 110

TGTGCTCCCCCAGGCCCCTG AGCCTGCGACCTCCTGGCAC TGTTGGGAGTTCTCTAGGGG - 50

+1 (CAP site)

CAGGCTGAAGGGCAGCATCC GAACTCAGGTGCCCCTGCTC CCAACGCCT TGTCCCCCA

+21

GGCTCACTCACC ATG ACC AAG CTG AGC GCC CAA GTC AAA GGC TCT CTC AAC AT

+80

C ACC ACC CCG GGG CTG CAG *GTCGAC*

FIG. 4'B

PRV.STRIDER -> Restriction Map

DNA sequence 1991 b.p. GTCGACCTGCAG ... CTGCAGGTCGAC linear

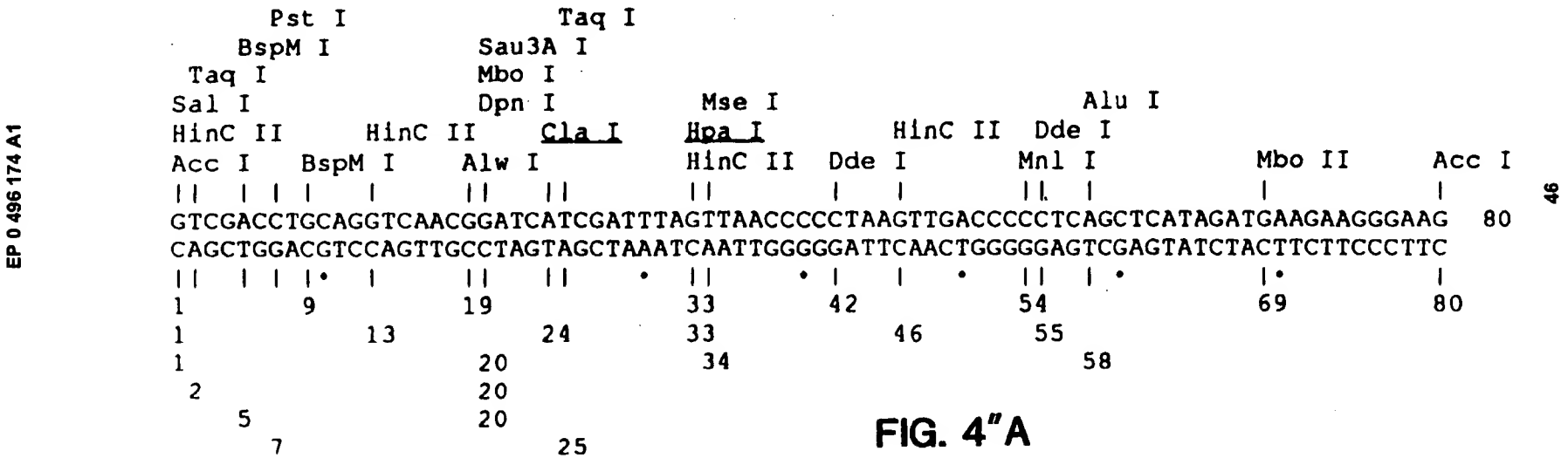


FIG. 4"A

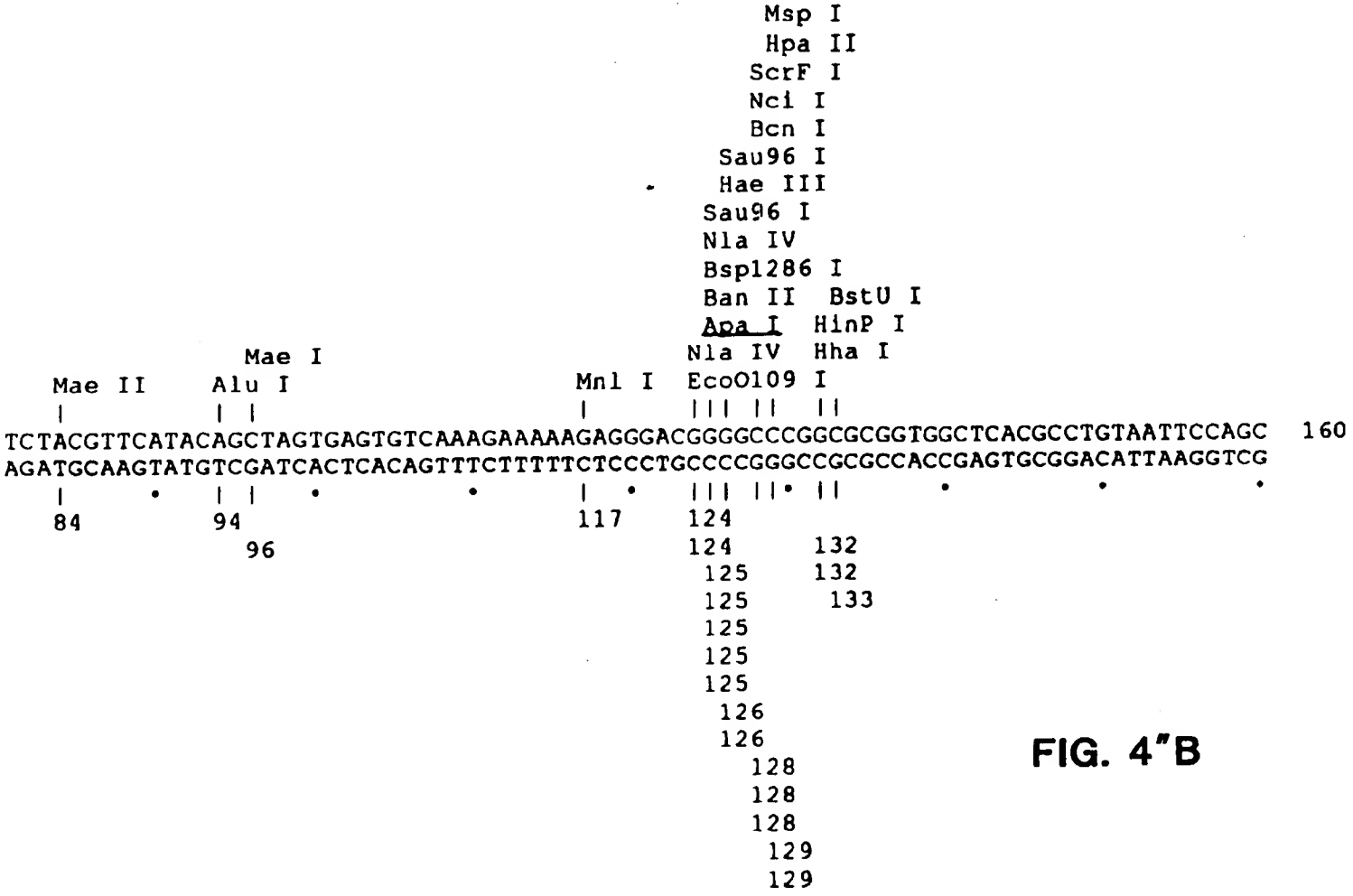

Msp I
Hpa II
ScrF I
Nci I
Bcn I
Sau96 I
Hae III
Sau96 I
Nla IV
Bsp1286 I
Ban II BstU I
Apa I HinP I
Nla IV Hha I
Mae I
Mae II Alu I Mnl I EcoO109 I
TCTACGTTCATACAGCTAGTGAGTGTCAAAGAAAAAGAGGGACGGGGCCCGGCGCGGTGGCTCACGCCTGTAATTCCAGC 160
AGATGCAAGTATGTCGATCACTCACAGTTTCTTTTTCTCCCTGCCCCGGGCCGCGCCACCGAGTGCGGACATTAAGGTCG
84 94 117 124
96 124 132
125 132
125 133
125
125
125
126
126
128
128
128
129
129

FIG. 4"B

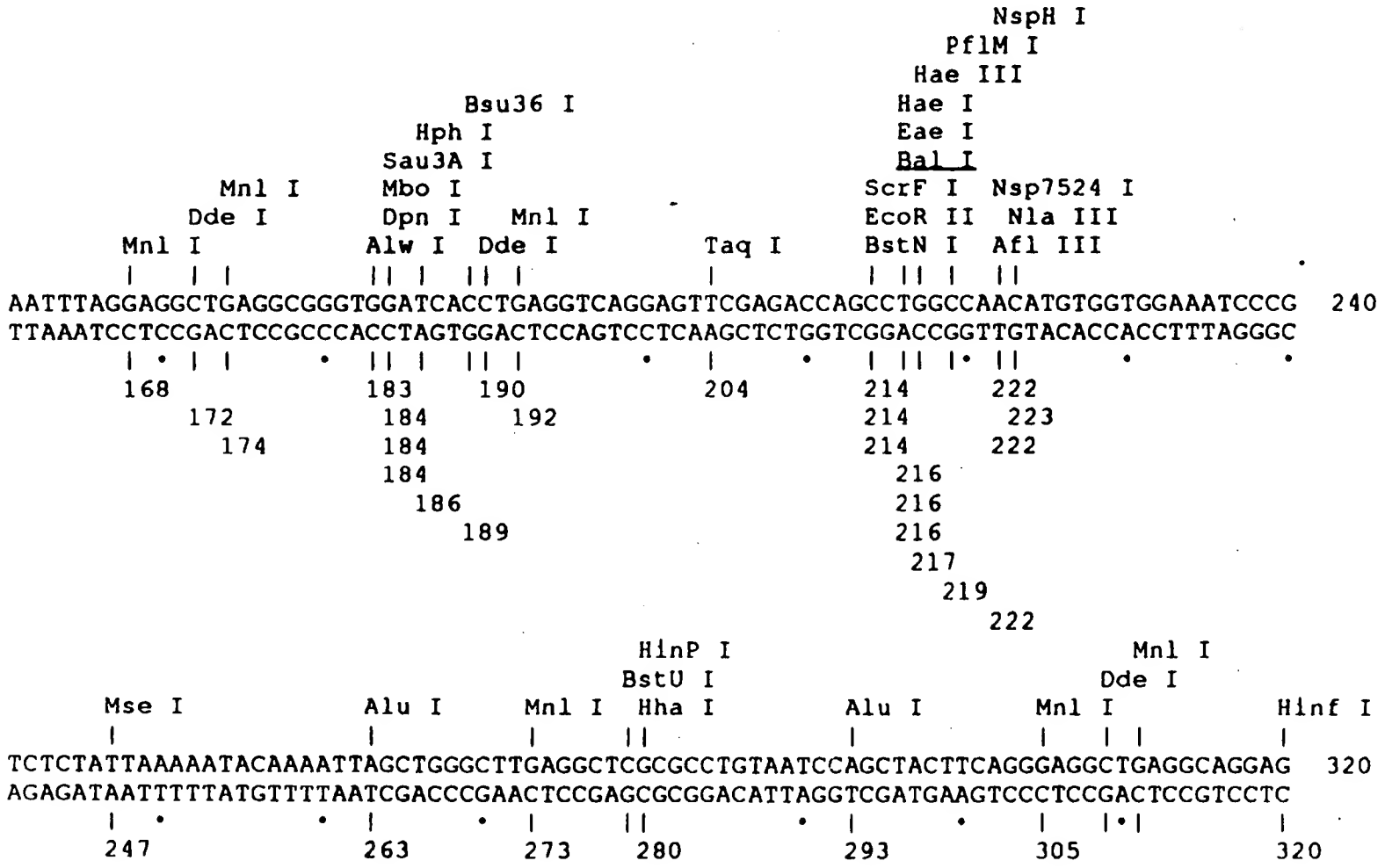

NspH I
PflM I
Hae III
Bsu36 I
Hae I
Hph I
Eae I
Sau3A I
Bal I
Mnl I
Mbo I
ScrF I
Nsp7524 I
Dde I
Dpn I
Mnl I
EcoR II
Nla III
Mnl I
Alw I
Dde I
Taq I
BstN I
Afl III
AATTTAGGAGGCTGAGGCGGGTGGATCACCTGAGGTCAGGAGTTCGAGACCAGCCTGGCCAACATGTGGTGGAAATCCCG 240
TTAAATCCTCCGACTCCGCCCACCTAGTGGACTCCAGTCCTCAAGCTCTGGTCGGACCGGTTGTACACCACCTTTAGGGC
168
172
174
183
184
184
184
186
189
190
192
204
214
214
214
216
216
216
217
219
222
214
222
223
222
HinP I
BstU I
Mnl I
Dde I
Mse I
Alu I
Mnl I
Hha I
Alu I
Mnl I
Hinf I
TCTCTATTAAAAATACAAAATTAGCTGGGCTTGAGGCTCGCGCCTGTAATCCAGCTACTTCAGGGAGGCTGAGGCAGGAG 320
AGAGATAATTTTTATGTTTTAATCGACCCGAACTCCGAGCGCGGACATTAGGTCGATGAAGTCCCTCCGACTCCGTCCTC
247
263
273
280
293
305
320

FIG. 4"C

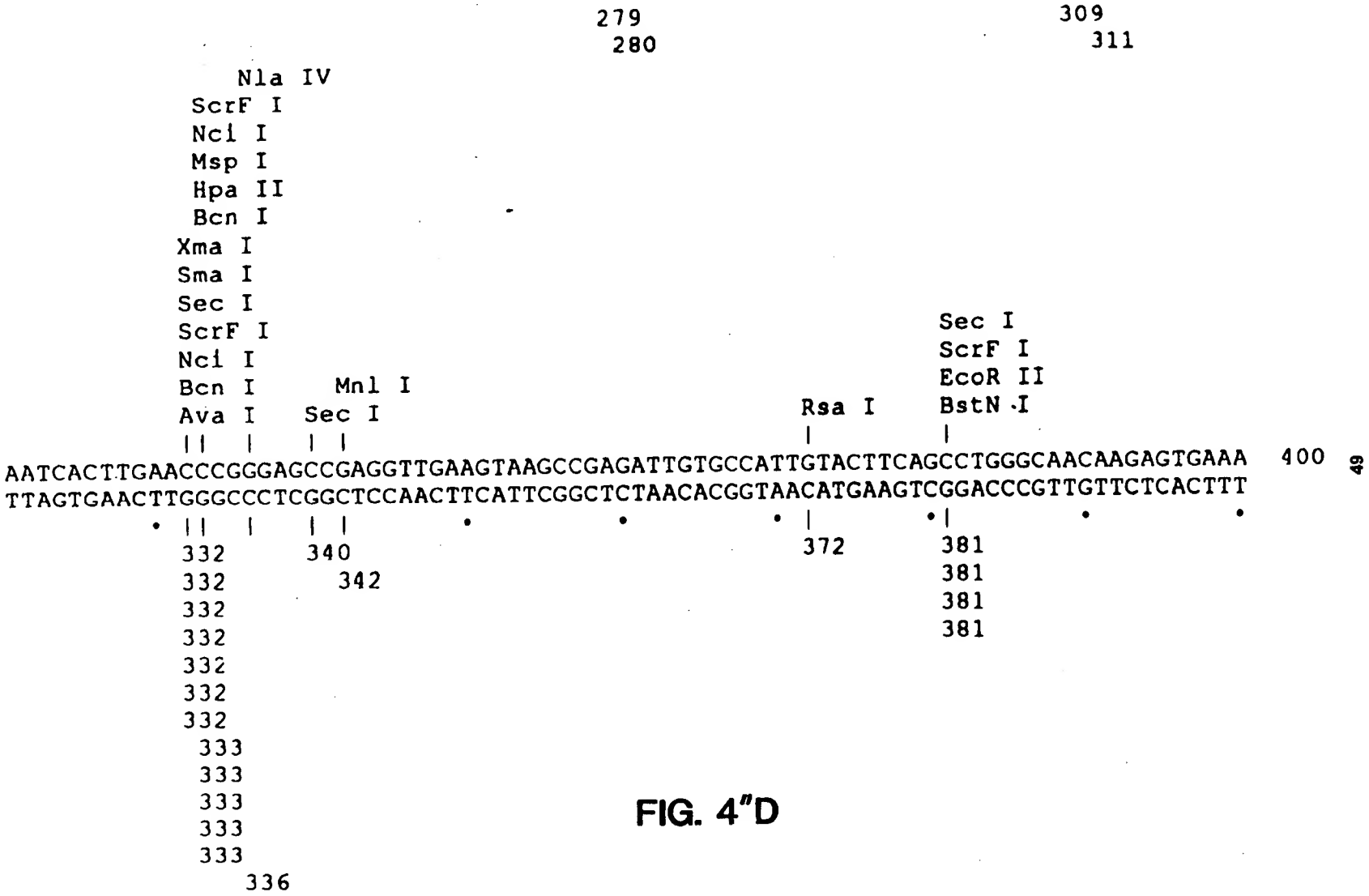
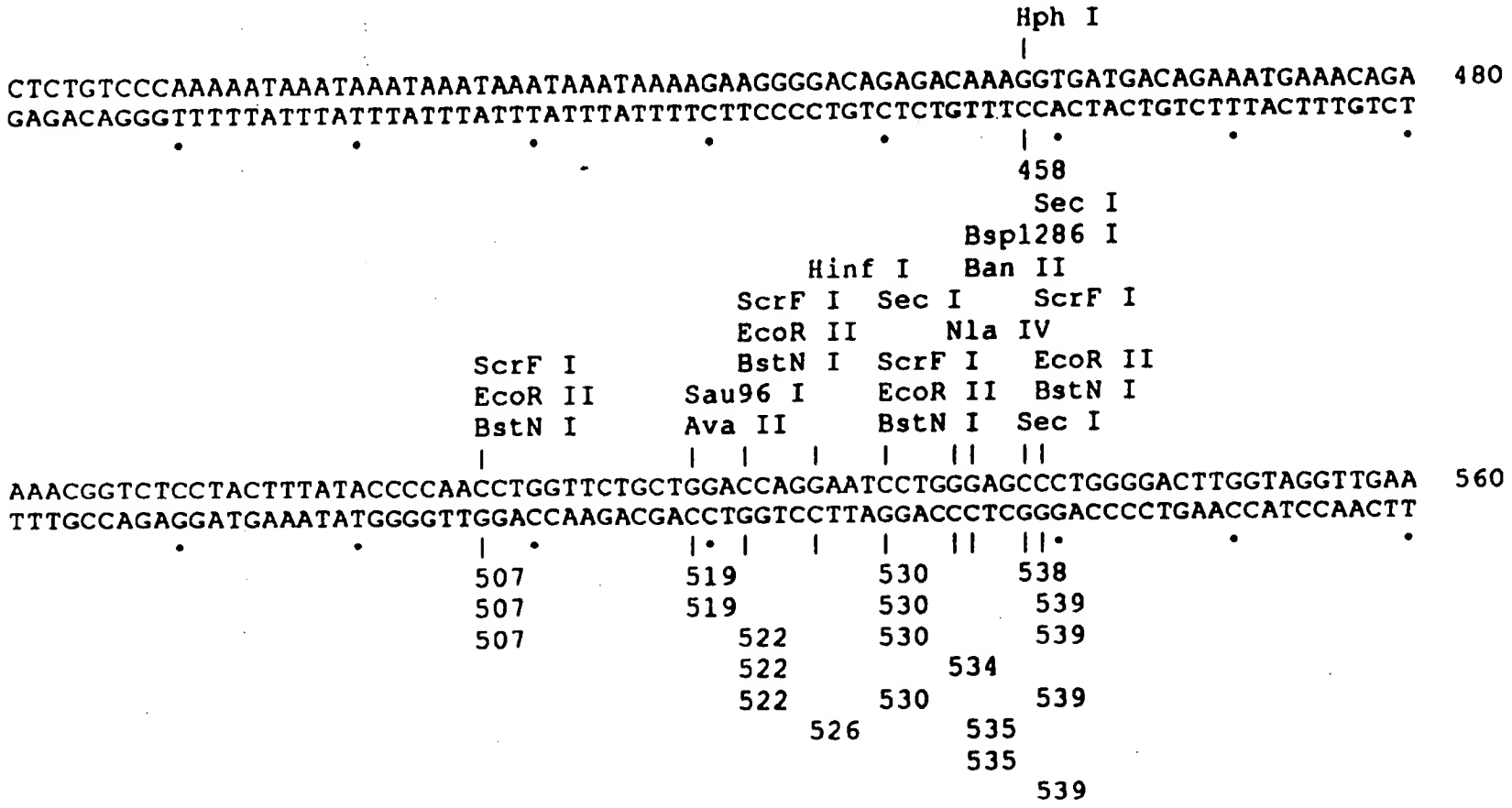

279
280
309
311
Nla IV
ScrF I
Nci I
Msp I
Hpa II
Bcn I
Xma I
Sma I
Sec I
ScrF I
Nci I
Bcn I
Ava I
Mnl I
Sec I
Rsa I
Sec I
ScrF I
EcoR II
BstN I
AATCACTTGAACCCGGGAGCCGAGGTTGAAGTAAGCCGAGATTGTGCCATTGTACTTCAGCCTGGGCAACAAGAGTGAAA 400
TTAGTGAACTTGGGCCCTCGGCTCCAACTTCATTCGGCTCTAACACGGTAACATGAAGTCGGACCCGTTGTTCTCACTTT
332
340
342
372
381
336

FIG. 4"D


Hph I
CTCTGTCCCAAAAATAAATAAATAAATAAATAAATAAAAGAAGGGGACAGAGACAAAGGTGATGACAGAAATGAAACAGA 480
GAGACAGGGTTTTTATTTATTTATTTATTTATTTATTTTCTTCCCCTGTCTCTGTTTCCACTACTGTCTTTACTTTGTCT
458
Sec I
Bsp1286 I
Hinf I
Ban II
ScrF I
Sec I
ScrF I
EcoR II
Nla IV
ScrF I
BstN I
ScrF I
EcoR II
EcoR II
Sau96 I
EcoR II
BstN I
BstN I
Ava II
BstN I
Sec I
AAACGGTCTCCTACTTTATACCCCAACCTGGTTCTGCTGGACCAGGAATCCTGGGAGCCCTGGGGACTTGGTAGGTTGAA 560
TTTGCCAGAGGATGAAATATGGGGTTGGACCAAGACGACCTGGTCCTTAGGACCCTCGGGACCCCTGAACCATCCAACTT
507
519
530
538
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526
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535
539

50

FIG. 4"E

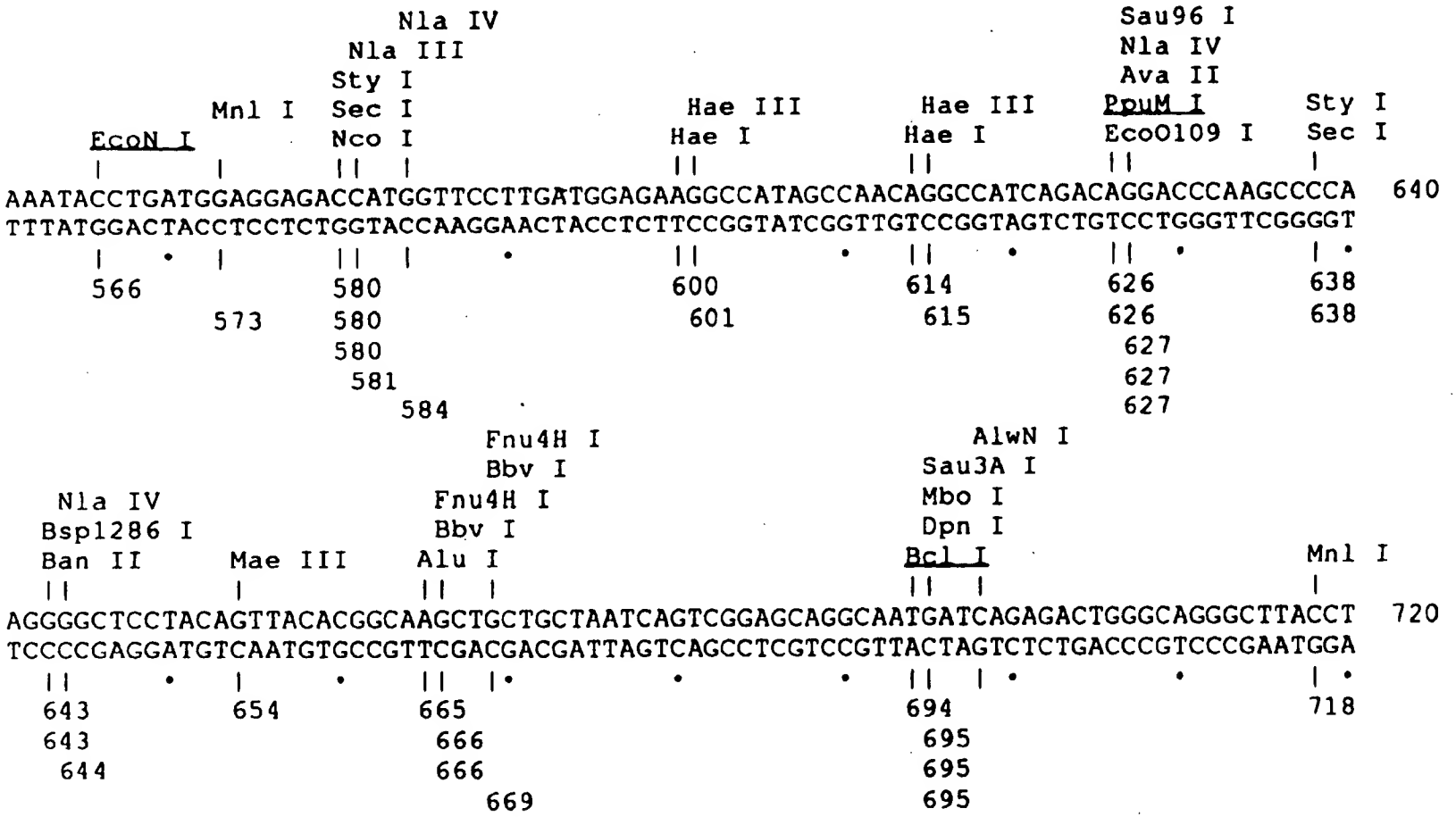
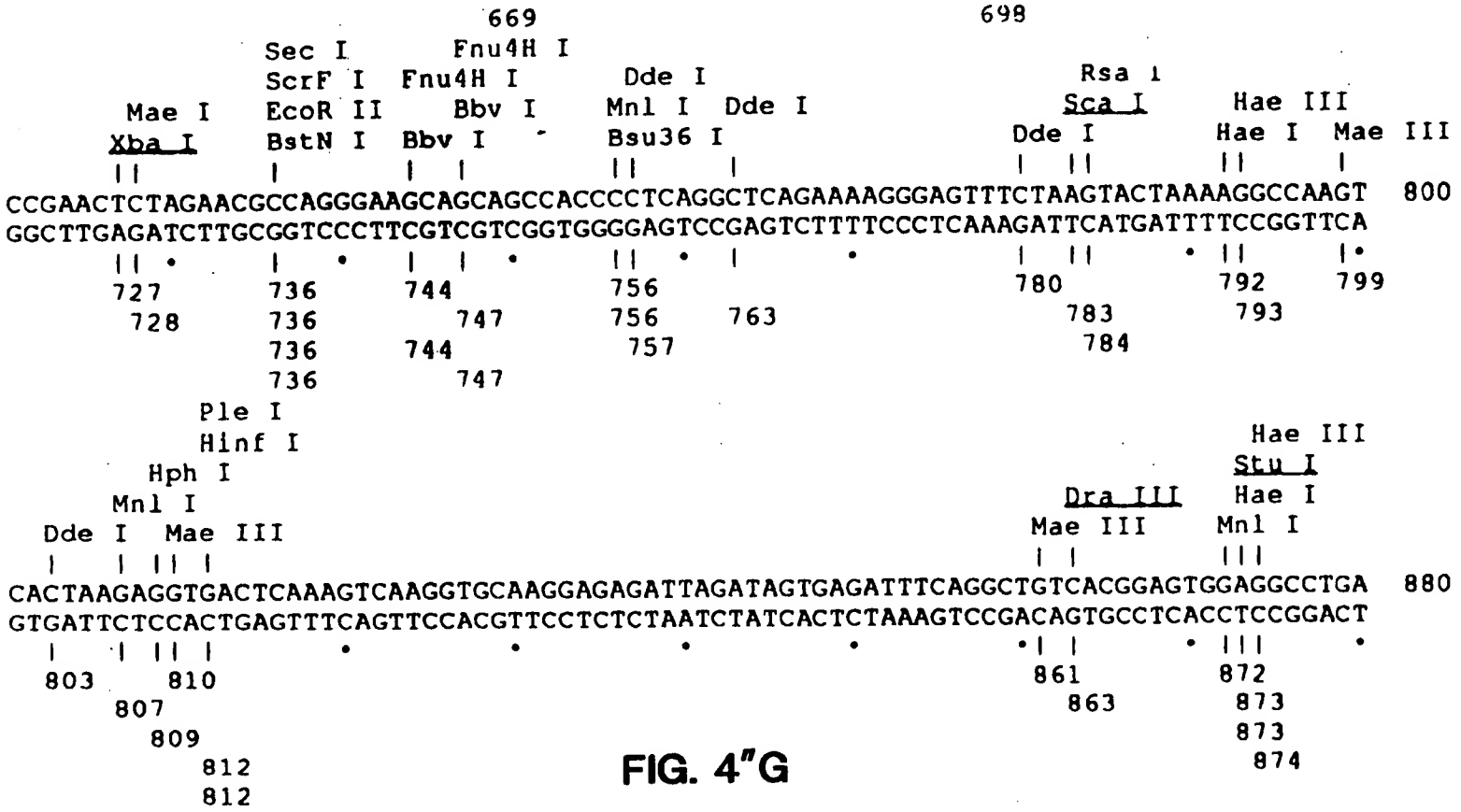

Nla IV
Nla III
Sty I
Sec I
Nco I
Mnl I
EcoN I
Hae III
Hae I
Hae III
Hae I
Sau96 I
Nla IV
Ava II
PpuM I
EcoO109 I
Sty I
Sec I
AAATACCTGATGGAGGAGACCATGGTTCCTTGATGGAGAAGGCCATAGCCAACAGGCCATCAGACAGGACCCAAGCCCCA 640
TTTATGGACTACCTCCTCTGGTACCAAGGAACTACCTCTTCCGGTATCGGTTGTCCGGTAGTCTGTCCTGGGTTCGGGGT
566 573 580 580 580 581 584 600 601 614 615 626 626 627 627 627 638 638
Fnu4H I
Bbv I
AlwN I
Sau3A I
Mbo I
Dpn I
Nla IV
Bsp1286 I
Ban II
Fnu4H I
Bbv I
Alu I
Mae III
Bcl I
Mnl I
AGGGGCTCCTACAGTTACACGGCAAGCTGCTGCTAATCAGTCGGAGCAGGCAATGATCAGAGACTGGGCAGGGCTTACCT 720
TCCCCGAGGATGTCAATGTGCCGTTCGACGACGATTAGTCAGCCTCGTCCGTTACTAGTCTCTGACCCGTCCCGAATGGA
643 643 644 654 665 666 666 669 694 695 695 695 718

FIG. 4"F


669
698
Sec I
Fnu4H I
ScrF I
Fnu4H I
Dde I
Rsa I
Mae I
EcoR II
Bbv I
Mnl I
Dde I
Sca I
Hae III
Xba I
BstN I
Bbv I
Bsu36 I
Dde I
Hae I
Mae III
CCGAACTCTAGAACGCCAGGGAAGCAGCAGCCACCCCTCAGGCTCAGAAAAGGGAGTTTCTAAGTACTAAAAGGCCAAGT 800
GGCTTGAGATCTTGCGGTCCCTTCGTCGTCGGTGGGGAGTCCGAGTCTTTTCCCTCAAAGATTCATGATTTTCCGGTTCA
727
736
744
756
780
792
799
728
736
747
756
763
783
793
736
744
757
784
736
747
Ple I
Hinf I
Hae III
Hph I
Stu I
Mnl I
Dra III
Hae I
Dde I
Mae III
Mae III
Mnl I
CACTAAGAGGTGACTCAAAGTCAAGGTGCAAGGAGAGATTAGATAGTGAGATTTCAGGCTGTCACGGAGTGGAGGCCTGA 880
GTGATTCTCCACTGAGTTTCAGTTCCACGTTCCTCTCTAATCTATCACTCTAAAGTCCGACAGTGCCTCACCTCCGGACT
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863
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809
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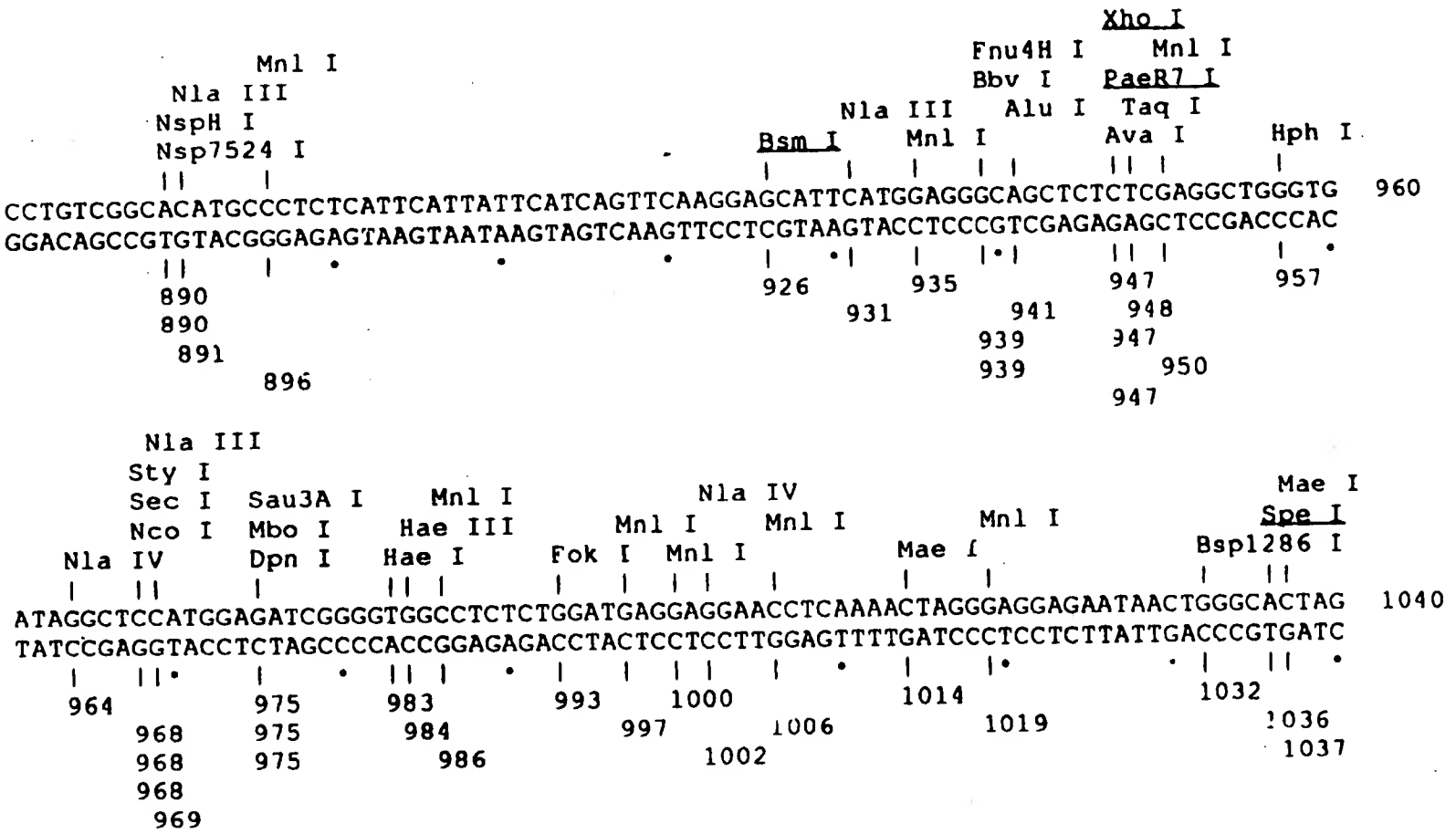
874
812

FIG. 4"G


Xho I
Fnu4H I
Mnl I
Bbv I
PaeR7 I
Mnl I
Nla III
Alu I
Taq I
NspH I
Nsp7524 I
Bsm I
Mnl I
Ava I
Hph I
CCTGTCGGCACATGCCCTCTCATTCATTATTCATCAGTTCAAGGAGCATTCATGGAGGGCAGCTCTCTCGAGGCTGGGTG 960
GGACAGCCGTGTACGGGAGAGTAAGTAATAAGTAGTCAAGTTCCTCGTAAGTACCTCCCGTCGAGAGAGCTCCGACCCAC
926
935
947
957
890
890
891
931
941
948
939
947
939
950
896
947
Nla III
Sty I
Sec I
Nco I
Sau3A I
Mbo I
Dpn I
Mnl I
Hae III
Hae I
Nla IV
Mnl I
Fok I
Mnl I
Mnl I
Mae I
Mnl I
Mae I
Spe I
Bsp1286 I
ATAGGCTCCATGGAGATCGGGGTGGCCTCTCTGGATGAGGAGGAACCTCAAAACTAGGGAGGAGAATAACTGGGCACTAG 1040
TATCCGAGGTACCTCTAGCCCCACCGGAGAGACCTACTCCTCCTTGGAGTTTTGATCCCTCCTCTTATTGACCCGTGATC
964
975
983
993
1000
1014
1032
968
975
984
997
1006
1019
1036
968
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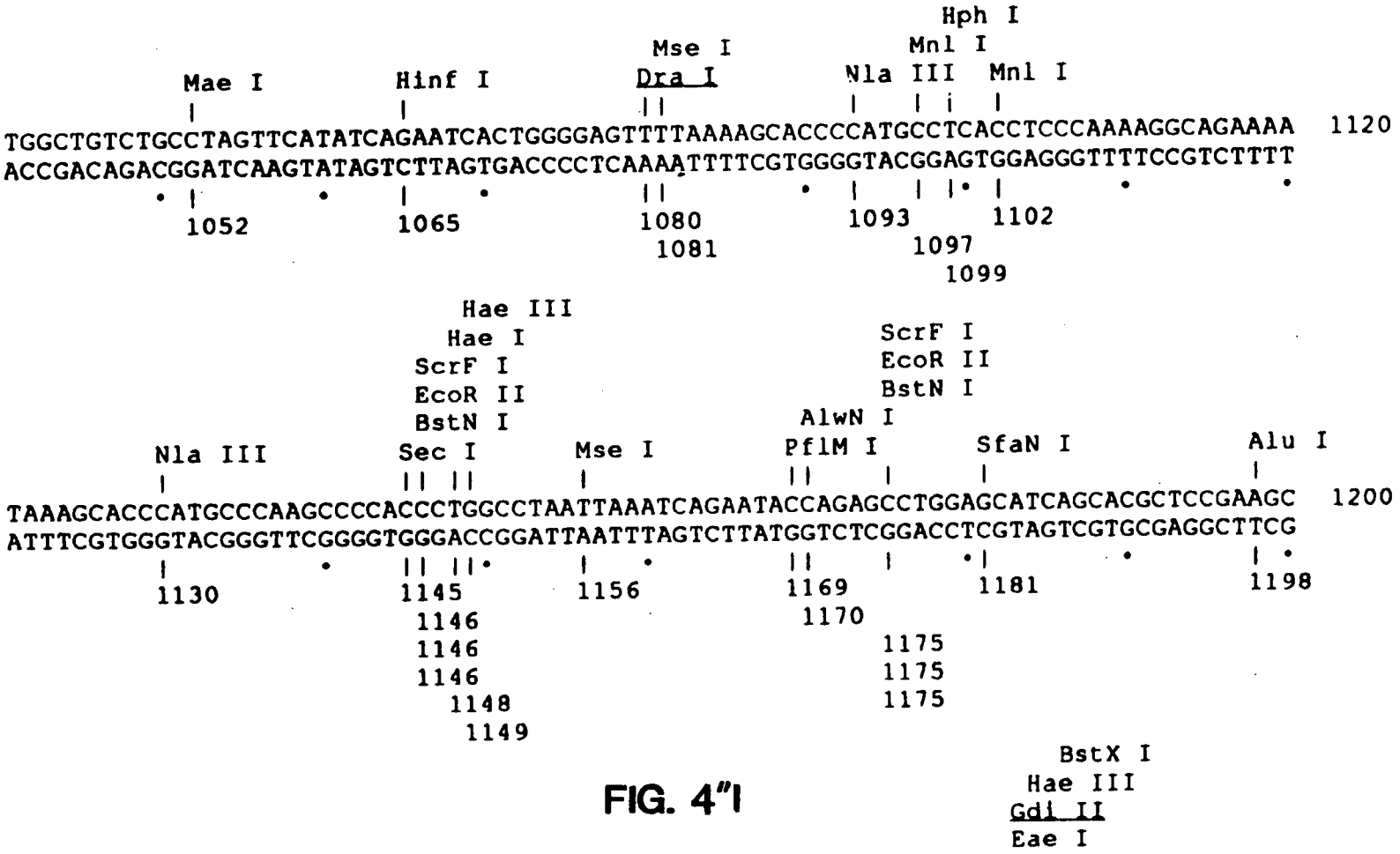
986
1002
1037
968
969

FIG. 4"H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Mae I
Hinf I
Dra I
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1052
1065
1080
1081
1093
1097
1099
1102
Hae III
Hae I
ScrF I
EcoR II
BstN I
ScrF I
EcoR II
BstN I
AlwN I
Nla III
Sec I
Mse I
PflM I
SfaN I
Alu I
TAAAGCACCCATGCCCAAGCCCCACCCTGGCCTAATTAAATCAGAATACCAGAGCCTGGAGCATCAGCACGCTCCGAAGC 1200
ATTTCGTGGGTACGGGTTCGGGGTGGGACCGGATTAATTTAGTCTTATGGTCTCGGACCTCGTAGTCGTGCGAGGCTTCG
1130
1145
1146
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1149
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1181
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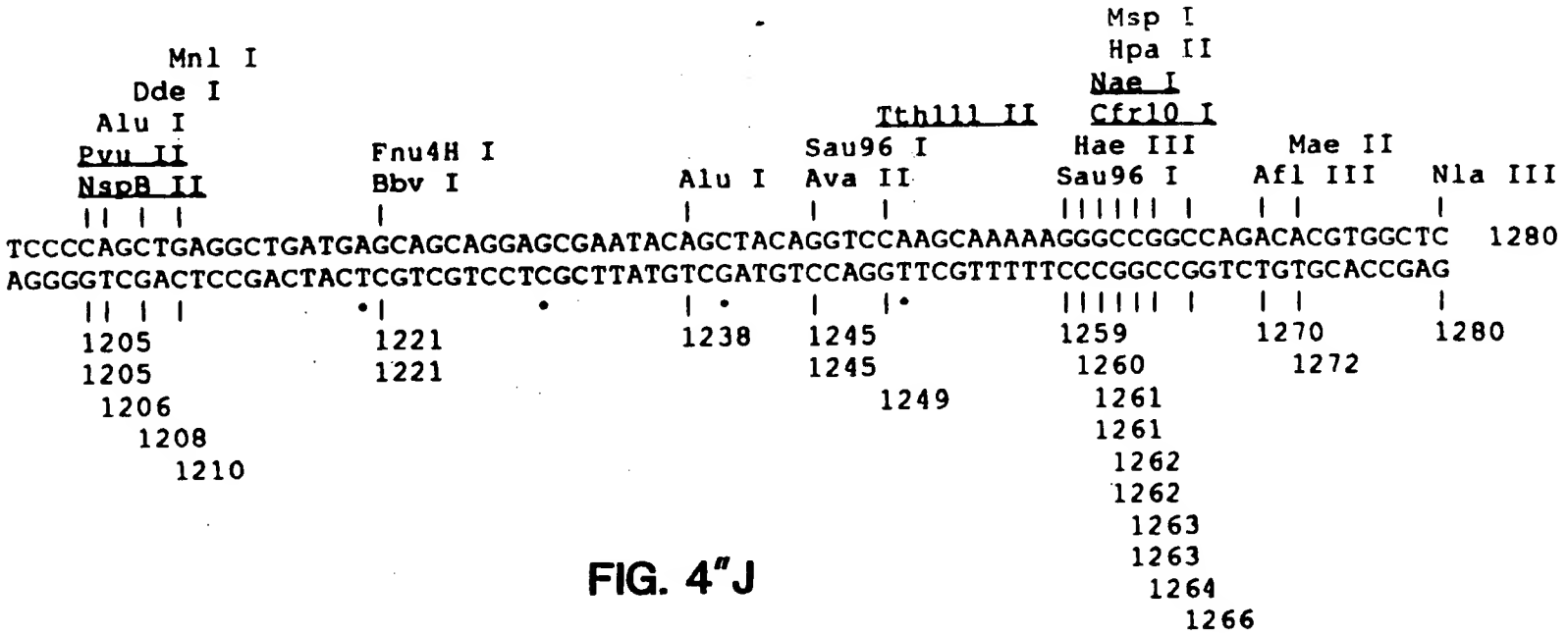
BstX I
Hae III
Gdi II
Eae I

FIG. 4"I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Dde I
Tth111 II
Cfr10 I
Alu I
Sau96 I
Hae III
Mae II
Pvu II
Fnu4H I
Alu I
Ava II
Sau96 I
Afl III
Nla III
NspB II
Bbv I
TCCCCAGCTGAGGCTGATGAGCAGCAGGAGCGAATACAGCTACAGGTCCAAGCAAAAAGGGCCGGCCAGACACGTGGCTC 1280
AGGGGTCGACTCCGACTACTCGTCGTCCTCGCTTATGTCGATGTCCAGGTTCGTTTTTCCCGGCCGGTCTGTGCACCGAG
1205
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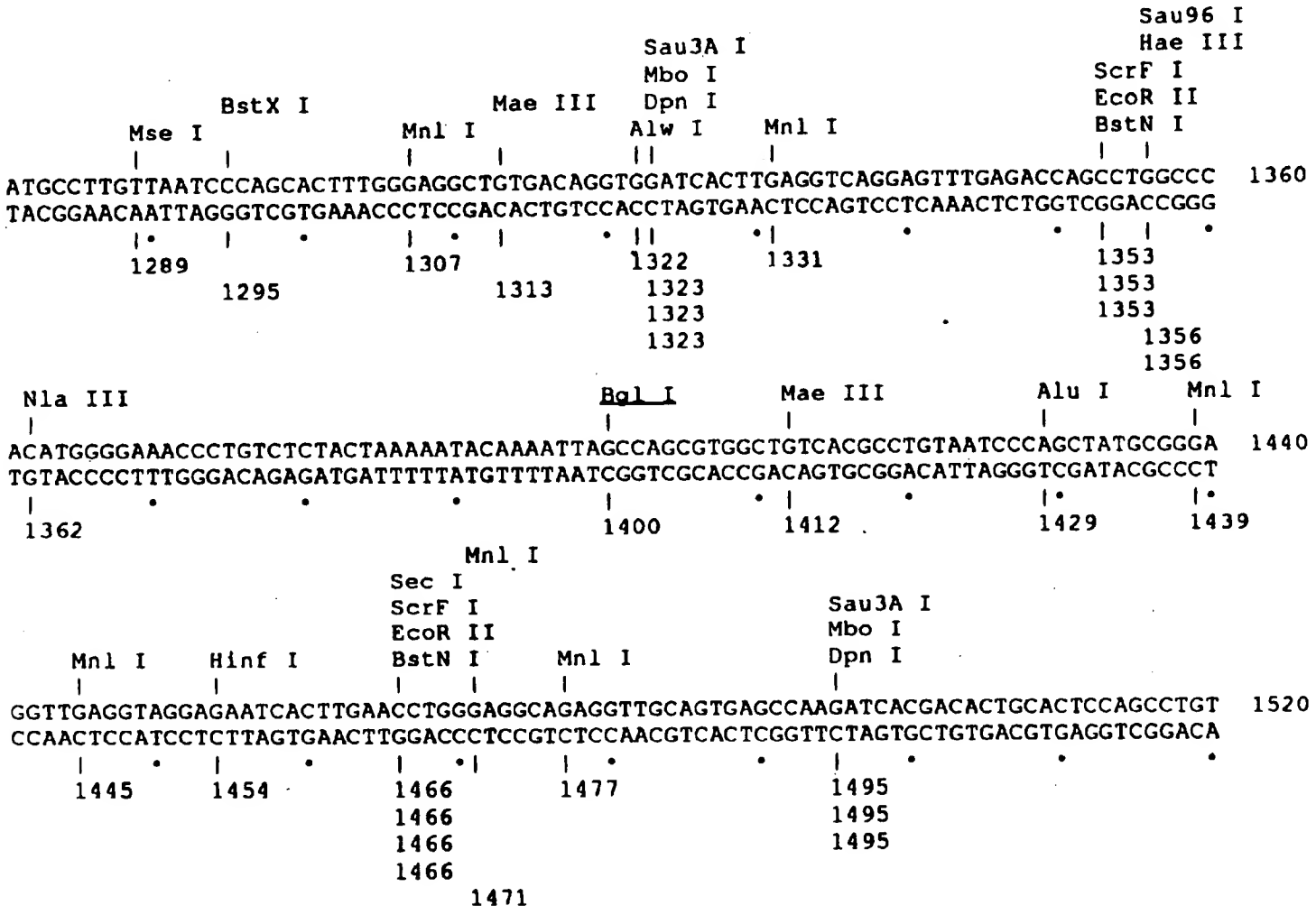
1266

FIG. 4"J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Hae III
Sau3A I
Mbo I
Dpn I
ScrF I
EcoR II
BstX I
Mae III
BstN I
Mse I
Mnl I
Alw I
Mnl I
ATGCCTTGTTAATCCCAGCACTTTGGGAGGCTGTGACAGGTGGATCACTTGAGGTCAGGAGTTTGAGACCAGCCTGGCCC 1360
TACGGAACAATTAGGGTCGTGAAACCCTCCGACACTGTCCACCTAGTGAACTCCAGTCCTCAAACTCTGGTCGGACCGGG
1289
1307
1322
1331
1353
1295
1313
1323
1353
1323
1353
1323
1356
1356
Nla III
Bgl I
Mae III
Alu I
Mnl I
ACATGGGGAAACCCTGTCTCTACTAAAAATACAAAATTAGCCAGCGTGGCTGTCACGCCTGTAATCCCAGCTATGCGGGA 1440
TGTACCCCTTTGGGACAGAGATGATTTTTATGTTTTAATCGGTCGCACCGACAGTGCGGACATTAGGGTCGATACGCCCT
1362
1400
1412
1429
1439
Mnl I
Sec I
ScrF I
EcoR II
Sau3A I
Mbo I
Mnl I
Hinf I
BstN I
Mnl I
Dpn I
GGTTGAGGTAGGAGAATCACTTGAACCTGGGAGGCAGAGGTTGCAGTGAGCCAAGATCACGACACTGCACTCCAGCCTGT 1520
CCAACTCCATCCTCTTAGTGAACTTGGACCCTCCGTCTCCAACGTCACTCGGTTCTAGTGCTGTGACGTGAGGTCGGACA
1445
1454
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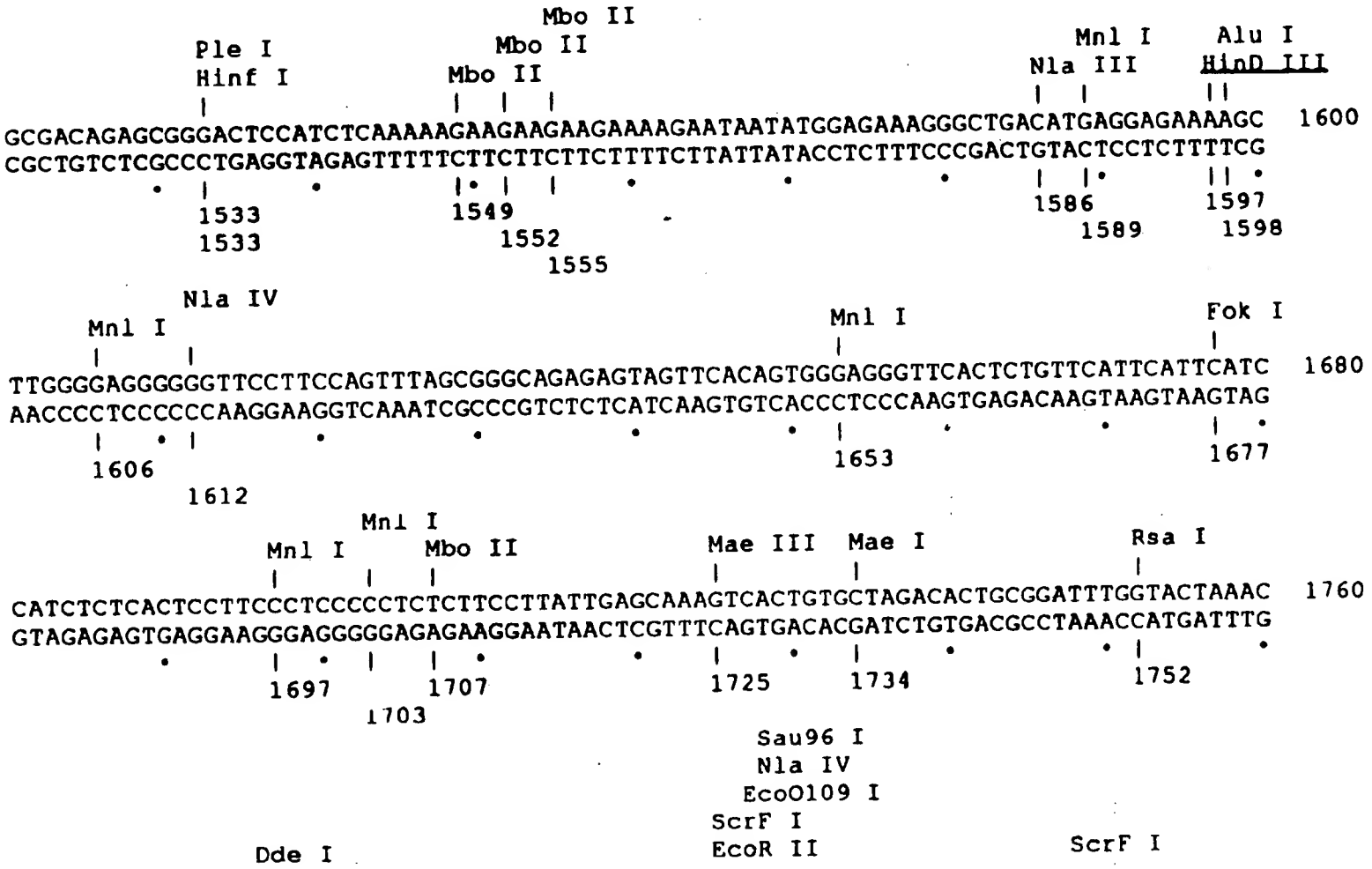
1477
1495
1466
1495
1466
1495
1466
1471

FIG. 4"K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Ple I
Mbo II
Hinf I
Mbo II
Mnl I
Nla III
Alu I
HinD III
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1533
1533
1549
1552
1555
1586
1589
1597
1598
Nla IV
Mnl I
Mnl I
Fok I
TTGGGGAGGGGGGTTCCTTCCAGTTTAGCGGGCAGAGAGTAGTTCACAGTGGGAGGGTTCACTCTGTTCATTCATTCATC 1680
AACCCCTCCCCCCAAGGAAGGTCAAATCGCCCGTCTCTCATCAAGTGTCACCCTCCCAAGTGAGACAAGTAAGTAAGTAG
1606
1612
1653
1677
Mnl I
Mnl I
Mbo II
Mae III
Mae I
Rsa I
CATCTCTCACTCCTTCCCTCCCCCTCTCTTCCTTATTGAGCAAAGTCACTGTGCTAGACACTGCGGATTTGGTACTAAAC 1760
GTAGAGAGTGAGGAAGGGAGGGGGAGAGAAGGAATAACTCGTTTCAGTGACACGATCTGTGACGCCTAAACCATGATTTG
1697
1703
1707
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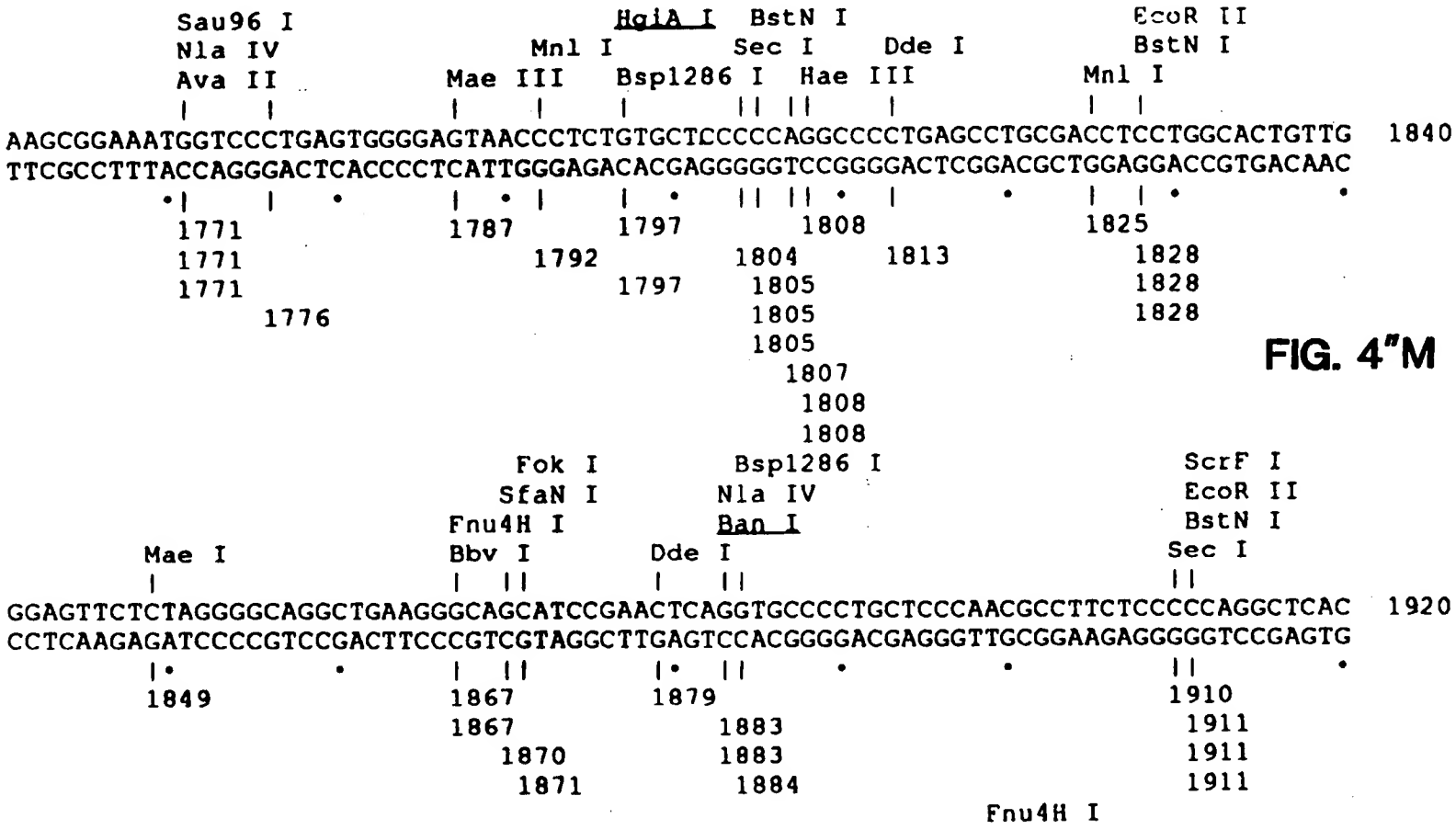
1734
1752
Sau96 I
Nla IV
EcoO109 I
ScrF I
EcoR II
Dde I
ScrF I

FIG. 4"L


Sau96 I
Nla IV
Ava II
HgiA I
BstN I
Mnl I
Sec I
Dde I
EcoR II
BstN I
Mae III
Bsp1286 I
Hae III
Mnl I
AAGCGGAAATGGTCCCTGAGTGGGGAGTAACCCTCTGTGCTCCCCCAGGCCCCTGAGCCTGCGACCTCCTGGCACTGTTG 1840
TTCGCCTTTACCAGGGACTCACCCCTCATTGGGAGACACGAGGGGGTCCGGGGACTCGGACGCTGGAGGACCGTGACAAC
1771
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1771
1792
1804
1813
1828
1771
1797
1805
1828
1776
1805
1828
1805
1807
1808
1808
Fok I
SfaN I
Fnu4H I
Bsp1286 I
Nla IV
Ban I
ScrF I
EcoR II
BstN I
Mae I
Bbv I
Dde I
Sec I
GGAGTTCTCTAGGGGCAGGCTGAAGGGCAGCATCCGAACTCAGGTGCCCCTGCTCCCAACGCCTTCTCCCCCAGGCTCAC 1920
CCTCAAGAGATCCCCGTCCGACTTCCCGTCGTAGGCTTGAGTCCACGGGGACGAGGGTTGCGGAAGAGGGGGTCCGAGTG
1849
1867
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1871
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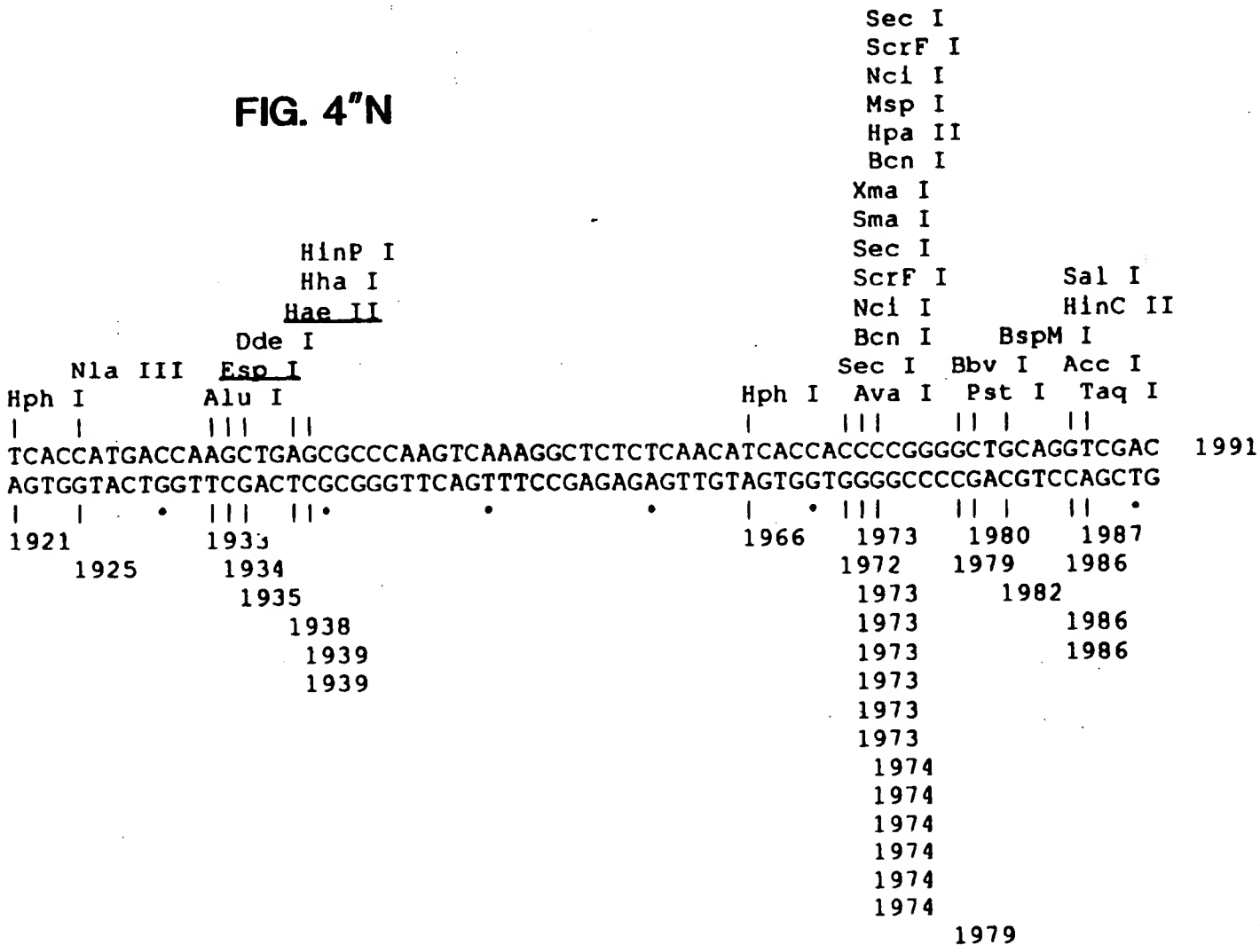
1911
Fnu4H I

FIG. 4"M

FIG. 4"N


Sec I
ScrF I
Nci I
Msp I
Hpa II
Bcn I
Xma I
Sma I
Sec I
ScrF I
Nci I
Bcn I
Sec I
HinP I
Hha I
Hae II
Dde I
Nla III
Esp I
Hph I
Alu I
Sal I
HinC II
BspM I
Bbv I
Acc I
Hph I
Ava I
Pst I
Taq I
TCACCATGACCAAGCTGAGCGCCCAAGTCAAAGGCTCTCTCAACATCACCACCCCGGGGCTGCAGGTCGAC
AGTGGTACTGGTTCGACTCGCGGGTTCAGTTTCCGAGAGAGTTGTAGTGGTGGGGCCCCGACGTCCAGCTG
1991
1921
1925
1933
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1938
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1972
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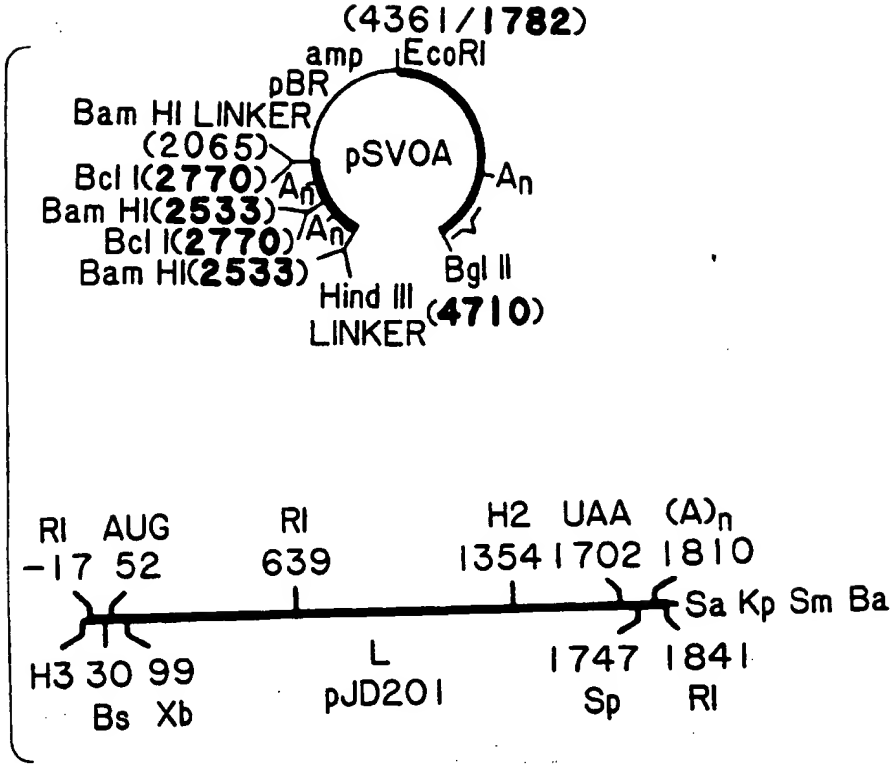
(4361/1782)
amp
EcoRI
pBR
Bam HI LINKER
(2065)
pSVOA
Bcl I(2770)
An
Bam HI(2533)
An
Bcl I(2770)
Bam HI(2533)
Bgl II
Hind III
LINKER
(4710)
RI AUG
-17 52
RI
639
H2 UAA (A)n
1354 1702 1810
Sa Kp Sm Ba
H3 30 99
Bs Xb
L
pJD201
1747 1841
Sp RI

FIG. 5

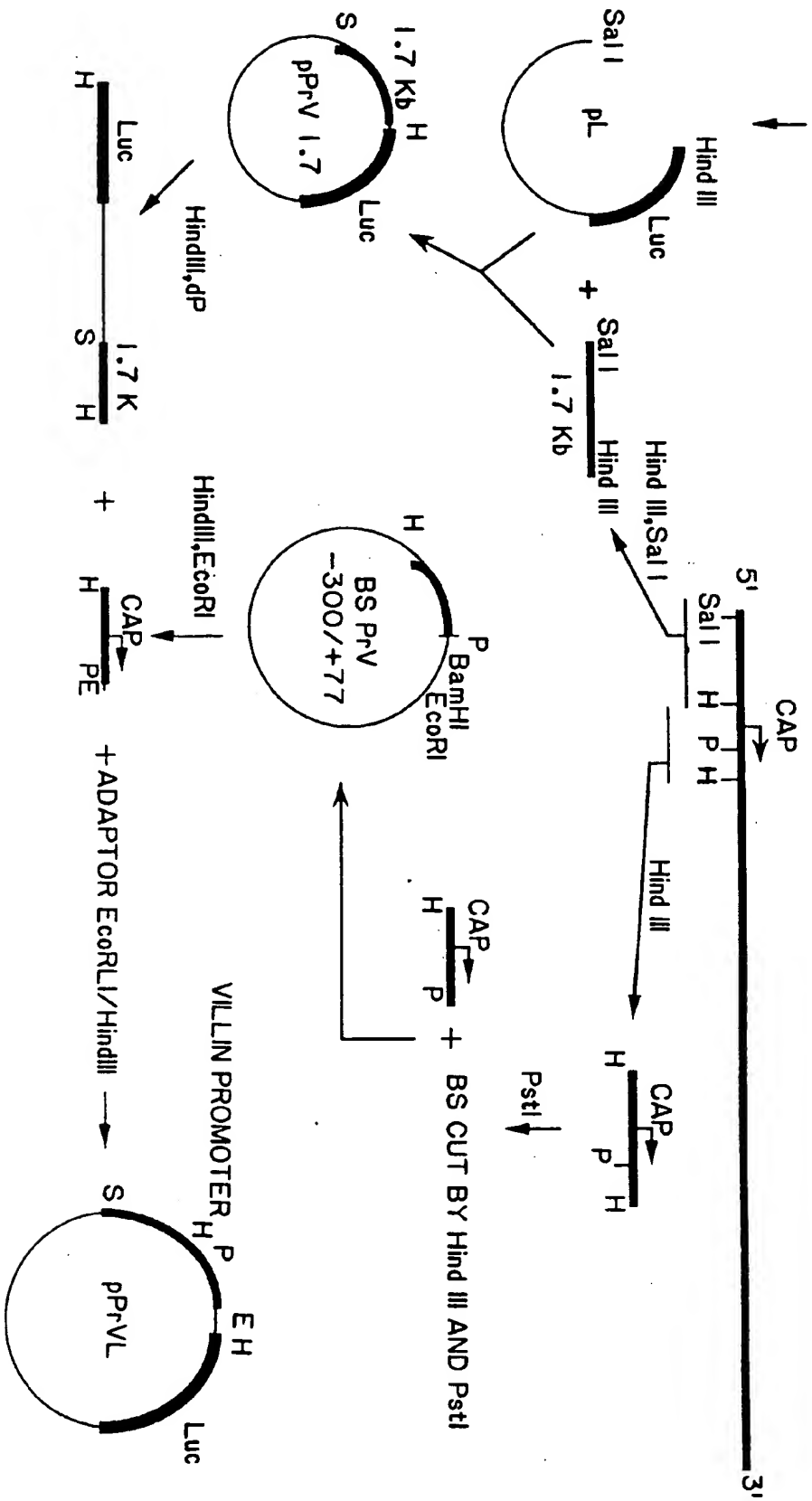
pSVOAL
Hind III
Sal I
pL
Luc
+
Sal I
1.7 Kb
Hind III
Hind III,Sal I
5'
Sal I
H
P
H
CAP
3'
Hind III
H
CAP
P
H
PstI
S
1.7 Kb H
pPrV 1.7
Luc
H
Luc
HindIII,dP
S
1.7 K
H
H
BS PrV
-300/+77
P
BamHI
EcoRI
CAP
H
P
+
BS CUT BY Hind III AND PstI
HindIII,EcoRI
+
H
CAP
PE
+ADAPTOR EcoRLI/HindIII
VILLIN PROMOTER
S
H
P
E H
pPrVL
Luc

FIG. 6

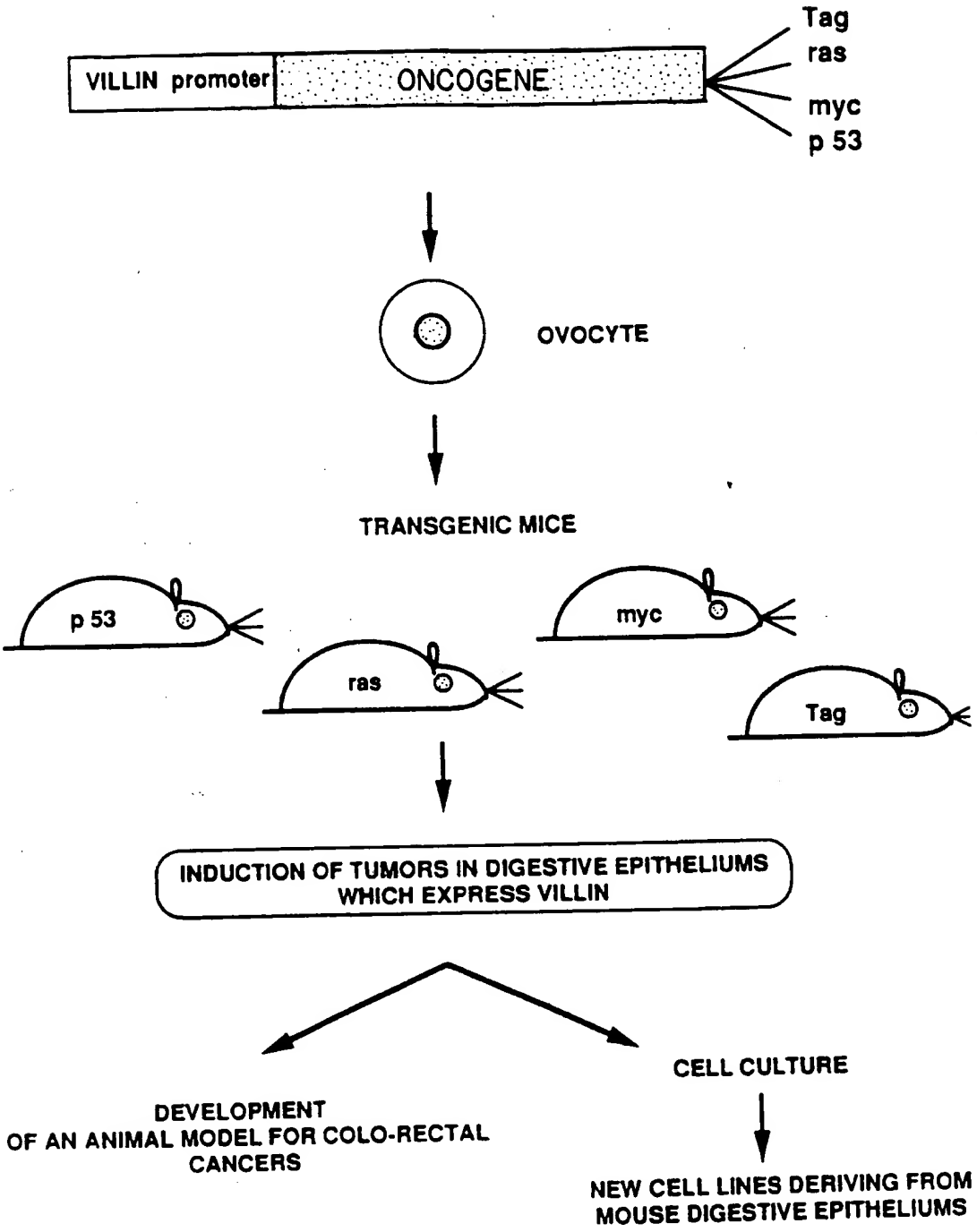
VILLIN promoter
ONCOGENE
Tag
ras
myc
p 53
OVOCYTE
TRANSGENIC MICE
p 53
ras
myc
Tag
INDUCTION OF TUMORS IN DIGESTIVE EPITHELIUMS WHICH EXPRESS VILLIN
DEVELOPMENT OF AN ANIMAL MODEL FOR COLO-RECTAL CANCERS
CELL CULTURE
NEW CELL LINES DERIVING FROM MOUSE DIGESTIVE EPITHELIUMS

FIG. 7

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 91 40 2887

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
	No relevant documents disclosed -----		C12N15/00 C12N15/85 C12N15/12 A07K67/027
			TECHNICAL FIELDS SEARCHED (Int. Cl.5) C12N C07K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	24 FEBRUARY 1992	CHAMBONNET F.J.

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